VOLUME I

NUCLEIC ACID
OUTLINES

VAN R. POTTER
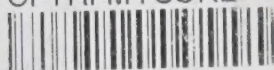
Dedication

This book is dedicated to the memory of
Richard Louis Potter (1918-1960).
His death will be a loss to all who read
this book.

Burgess

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BIOCHEMISTRY - MICROBIOLOGY

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NUCLEIC ACID OUTLINES

Volume I

STRUCTURE AND METABOLISM

by

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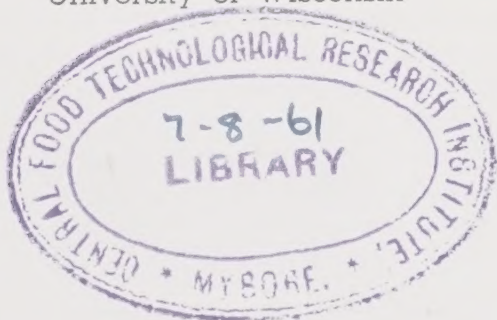
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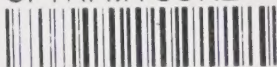
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PREFACE

These OUTLINES have been prepared as an adjunct to a course entitled "Nucleic Acids, Chemistry and Function" which the author has presented since 1955 at the University of Wisconsin. The course is open to graduate students who have had a beginning course in bio-chemistry, and each year there have been representatives from about 15 different departments in the Colleges of Agriculture, Letters and Science, and Medicine. The course is given in two lectures a week for nine weeks, and the 18 lectures are divided into nine on Chemistry and Metabolism and nine on Function and Applications. For the purposes of the course, the 18 lectures have been organized into 18 chapters of which the first nine are being published as Volume I, and it is expected that the second nine will appear as Volume II.

The OUTLINES are intended to enable the student to read the literature more purposefully in those areas in which he is particularly interested and to by-pass the literature in those areas that may be of secondary interest. An attempt has been made to provide specific references to original publications wherever possible. In order to limit the bibliographies to workable length, many excellent papers have not been referred to. Selection of references was not based on rigid criteria, but in most cases reflects the author's personal awareness or lack of awareness of available publications, as well as the author's interest in the subject. In many cases a personal publication may have been referred to when another publication would have been more suitable. In some cases the first announcement of a discovery has been mentioned, while in others the most recent paper has been cited, especially when it was felt that an excellent summary of previous literature was thereby made available.

During the past two years each student has been asked to submit one current reference of quality for each lecture, in the form of an abstract on one 8-1/2 x 11 sheet and a citation on a 3 x 5 card. The results have been interesting in that there was remarkably little duplication. Current

references were defined as those bearing the date of the current calendar year. Each year, there were approximately 50 students and 500 literature citations as compared with 50 x 18 or 900 references if no duplication had occurred, or 18 references if all chose the same references. The resulting cards and abstracts are filed for class use, and have been helpful in the present effort.

The OUTLINES are not intended to serve as a substitute for the excellent reference volumes by Chargaff and Davidson (Chargaff, E. and Davidson, J. N. , "The Nucleic Acids", volumes I and II, Academic Press, New York, 1955; Volume III in preparation, 1959-60; references to these works will be simplified by referring to the authors and volume numbers only). Many other reference works are referred to in the various chapters but no claim for all-inclusiveness is made. In general the rule has been that even a single reference is better than no reference at all.

Throughout the text the student will find references to the names of investigators and it emphasized that names are the keys to the literature. If the student learns to associate certain names with certain current investigations he will find that he can keep abreast of developments much more effectively than if he attempts to keep a mental subject index. It is always easier to find a paper by looking in an author index than it is to look in a subject index, as most older investigators probably will agree.

The present volume of NUCLEIC ACID OUTLINES is intended to provide a minimal background and a handy reference guide to the basic biochemistry of the nucleic acids. Essentially no material is presented on the organic chemistry, chemical syntheses and proofs of structure as exemplified by the excellent work of Todd and of Khorana. Similarly the book cannot serve as a guide to the physical chemistry of the nucleic acids. Perhaps these deficiencies can be corrected at some future date, but the present situation is one of urgency, in which it has become impossible to present in lecture form the material which is included

in the present volume, without some unified study material. It is felt that the function of the nucleic acids can be presented much more readily if the chemistry and metabolism is available in a unified text written specifically for graduate students.

The proposed chapter headings of the second volume are based on the lectures that constitute the course referred to above, and are listed after the chapter headings for Volume I. They include such topics as the cancer problem, the mechanism of radiation damage, adaptation, and such philosophical problems as the origin of life and the concept of human progress. Throughout the civilized world, thoughtful people in every discipline are beginning to feel an inner restlessness, a conviction that technology is not enough, that material welfare alone is not the key to the problems of mankind. There are some who feel that the problems of mankind can be solved in complete ignorance of events at the molecular level, while there are others who regard any analysis of man's dilemma as superficial if it fails to recognize the molecular basis of his existence, and the role of random events in determining the fate of individual lives. The author is of the opinion that all knowledge is relevant to a discussion of man and his future, and he is painfully aware of his own limitations. It is believed that no one possesses enough knowledge to write authoritatively the chapters proposed for volume II of this outline, but an attempt needs to be made, and the present volume is regarded as the necessary spade work for the lectures to follow. It may be useful in serving as the foundation for others who have entirely different ideas about what the subsequent nine lectures should contain.

Van R. Potter

Madison, Wisconsin
July 10, 1960

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Chapter I
Historical Introduction
to the Nucleic Acids

The history of any branch of science is usually repugnant to the beginner but a subject of increasing fascination for the experienced investigator. The beginner sees names as words in a foreign idiom, while the veteran sees names on a title page, firmly attached to a well-remembered investigation that set off a whole series of additional studies, or he sees a name and recalls the face, the voice and the mind that make it unique.

In this chapter and in the chapters to follow, names are considered important because it is emphasized that individuals are important. In any discussion of the role of the individual in science, there are two main viewpoints. One is that of determinism, which is the view that any discovery that is ever made would inevitably have been made regardless of the existence of any particular individual. The other viewpoint is that each individual is unique, not only in his potential, but also in his accumulated experience, and that each discovery, at the time of its occurrence, could have been made by a very few individuals. In this book, it will be emphasized again and again that all simple explanations must be viewed with suspicion, and here we come to the first example. The phenomenon of discovery does not depend on determinism alone, or on the individual alone: each discovery has a certain minimum requirement of accumulated knowledge and technique which have to be at the disposal of the individual before he can make a "discovery". In addition, the remainder of the community must have a certain level of sophistication to permit the discovery to be

recognized. In the present context, it will be argued that any student who wishes to become a scientist needs to develop an appreciation of the role of accumulated knowledge and technique in discovery, and he needs to develop a respect for the role of the individuals who have contributed to the events that are labeled discoveries. Most of these events were inevitable, and indeed, it is the business of science to proceed in such a way that discovery is inevitable. Most of us must devote our lives to the anonymous business of accumulating the material from which discoveries are made, without actually being a participant in the event labeled discovery. But the thrill of discovery is the thing that keeps scientists alive and it is a tremendous emotional experience regardless of whether the world nods approval or not. The essence of discovery is being the first to know, the first to see something that no one else has seen, the first to realize a relationship never before comprehended by anyone. It is the scientist's birthright, and anyone aspiring to a career in science would do well to inquire how discoveries are made. Although an element of luck is very important, no one would seriously argue against the idea that one of the elements of discovery is a sense of perspective. One must see each new event in relation to the accumulated relevant knowledge in the field, and if one is to make discoveries he must choose to work in an area and in a way that permits discovery. He must make use of the knowledge and tools that are available and he must anticipate the contributions that are going to be made by others in his field of work.

Finally it may be said that these obligations are not merely requirements for discovery. They are required by professional ethics. The scientist earns his living by making discoveries. He gets paid for making discoveries. If he is concerned merely with keeping busy, with doing work that has already been done, he is failing to meet his obligations to the society that makes his existence possible.

By these arguments we attempt to justify the mention of names in what is to follow.

THE PERIOD APPROACH TO
NUCLEIC ACID HISTORY

The goal of this chapter is to give the student a sense of perspective in the nucleic acid field, to relate events in this field to antecedent and parallel developments in related fields. One way to grasp history is to break up the elapsed time into periods, using particular dates that seem to be reasonable as the end of one era and the beginning of another. The number of periods must not be too great or too few, and they must correspond to easily grasped concepts of beginning, activity, and end. We prefer to compare the history of nucleic acid metabolism to growth of a bacterial culture. First there is a period corresponding to the preparation of the medium. This is followed by the period of inoculation, not a single event as in a bacterial culture but a period in which the initial discovery is certified and generally recognized as a discovery. The third period corresponds in a way to the induction period in a culture. Scientifically, this is a period of struggle, of wrong guesses, of tooling up, and a period during which new techniques are needed. The fourth period to be described corresponds to the period of logarithmic growth. It is a time when each new discovery is related to so many existing facts that a whole new crop of discoveries follow and set up conditions for further discoveries, with the actual volume of data outpacing the capacity for comprehension. This period in the nucleic acid field extends into the present and no one can predict how long it will continue.

The period approach is of course entirely arbitrary and is mainly a matter of convenience in attempting to make the picture as easy as possible for the student to grasp and retain.

I. *THE PRECONDITIONS FOR DISCOVERY. (1775-1869)*

The choice of 1775 as the first date in this historical survey might not coincide with what would be considered appropriate by either Chargaff or Davidson but we hasten to point out that while it has the virtues of a landmark

in other contexts (George Washington and all that) for
students whose childhood was spent in the U. S. A., it
is also an important date in biochemical history. The
preconditions for discovery of the nucleic acids may be
conveniently categorized as chemical and biological in
this early period, and a few relevant landmarks will be
outlined.

A. Chemical landmarks

1775: Oxygen was shown by Lavoisier to be con-
sumed by animals and it was shown that CO_2
was given off. The old phlogiston theory had
just been overthrown by Priestly and oxygen
had just been discovered.

1776: Uric acid[6] was discovered by Scheele and by
Bergmann. It was the first purine to be iso-
lated owing to its occurrence as free crystals
in bird excreta and some samples of human
urine. Its cyclic structure was not proposed
until about 100 years later.

1817: Xanthine[6] was discovered in bladder stones
by Marcet. It was the second purine.

1844: Guanine[6] was discovered in bird excreta by
Magnus. It was the third purine and the first
nucleic acid component to be discovered,
some 25 years before nucleic acids were
known. Note that the name derives from
guano, the important fertilizer that consists
of dried bird excreta as found in certain is-
lands off the coast of Peru.

1850: Hypoxanthine[6] was isolated from beef spleen
by Scherer. It probably was a breakdown
product formed from adenylic acid.

1866: The ring structure of benzene was proposed
by Kekule. This date provides some per-
spective in that the ring structure of the pu-
rines was easier to imagine after this event.

1820-1869: The first amino acids[59] were discovered.
These included glycine (1820), leucine (1820),
tyrosine (1846), serine (1865), glutamic acid
(1866), aspartic acid (1868).

B. *Biological landmarks* [49]

1660:   The concept of the cell as a unit of structure
        was introduced by Robert Hooke, who first
        used the word cell in this connection.

1831:   The occurrence of nuclei in cells was noted
        by Robert Brown.

1838:   The occurrence of nucleoli within nuclei was
        noted by Schleiden.

1839:   The cell as a functional unit of life was pro-
        posed by Schwann and this concept, The Cell
        Theory, is generally credited to both
        Schleiden and Schwann. [49]

1858:   Rudolph Virchow[9] the father of pathology, ex-
        pressed the idea that all cells come from pre-
        existing cells, and further that cells are the
        basis of all pathology.

1858:   Charles Darwin[4, 10, 19, 23, 27, 48] announced the
        theory of the origin of species and the con-
        cepts of evolution in terms of mutation and
        natural selection, although at that time
        Mendelian genetics was unknown, and no hints
        as to the role or existence of the nucleic
        acids were available.

1861:   Max Schultze[49] expressed the cell concept in
        more or less modern form, that is, the cell
        as a unit consisting of a nucleus and its sur-
        rounding protoplasm.

1865:   Gregor Mendel[49] worked out the basic rules
        of heredity, but this work went unnoticed un-
        til 1900 and of course was not known or used
        by Miescher (below).

II. *THE PERIOD OF DISCOVERY AND CONSOLIDATION*
     (1869-1909)

A. *Discovery of nucleic acids by Miescher (1869)* [15]

The above landmarks in the chemical and biological
fields provide some insight into the scientific world

of Friedrich Miescher's day, and it is worth noting that in coming from Basel to study with Hoppe-Seyler in Tübingen in 1868-1869 he was in the very center of what was then a rather circumscribed world of biochemistry. Only a few of the facts listed above could have been unknown to Miescher when he chose to combine the tools of the biologist and the chemist in order to attempt a chemical characterization of cell nuclei. His source material was the discarded surgical bandages from the hospitals of his day, which the microscope showed to be loaded with pus cells. And so the story of the nucleic acids begins.

Miescher digested the pus cells with pepsin in the presence of hydrochloric acid and extracted the digest with ether. The nuclei remained in the aqueous phase, settled out by gravity and were collected by filtration. The material isolated was named "nuclein," since it came from nuclei. It was shown to be acidic in nature, insoluble in dilute acids but soluble in dilute alkali. It contained a considerable amount of phosphorus, which indicated that it was something new, because at that time the only known phosphorus compound in animal tissues was lecithin, which had been recently isolated in Hoppe-Seyler's laboratory.

Miescher's results were not accepted by Hoppe-Seyler who insisted on repeating the work himself before publishing it in the journal that he edited. Utilizing his prerogatives, he then published the original account and the confirmation in adjacent space. As a result, the new field was "certified" as soon as it was introduced and a new branch of biochemistry was established. Miescher made his discovery at the age of twenty-five and died at the early age of fifty-one in 1895.

Among his students were R. Altmann who developed methods for preparing protein-free nucleic acid and

first used the term, "nucleic acid", and <u>Piccard</u>,
who first isolated guanine and hypoxanthine from a
nucleic acid hydrolysate.

B. *Studies on nucleic acid components by Kossel (1878-
    1909)* [29]

Miescher's chief successor was another pupil of
Hoppe-Seyler, Albrecht Kossel, (1853-1927) who,
in turn, had many students who carried forward the
work on the nucleic acids.   These include Walter
Jones (1865-1935), of Johns Hopkins and P. A.
Levene (1869-1940) of the Rockefeller Institute.
These two investigators led the nucleic acid inves-
tigations in the U.S.A. for many years.   Kossel's
mature years were spent at the University of Mar-
burg (1895-1901), and at the University of Heidel-
berg (1901-1927).   "All of his students travelled to
Heidelberg with him, so that when he reached there,
he had a small group of graduate students which in-
creased until in 1905, according to Mathews, there
were thirty students from many lands."[29]   Among
the countries represented were England, the United
States, Russia and Japan, and it is undoubtedly true
that many of the students of today (including the
author) could trace their scientific lineage along
direct lines leading back to Kossel and to Hoppe-
Seyler, for Kossel's laboratory was not only the
center of nucleic acid chemistry, but of the chemis-
try of proteins, particularly the protamines and his-
tones.   Among his students in the protein field were
H. D. Dakin, E. B. Hart[20], A. P. Mathews, Otto
Folin, E. L. Kennaway, and many others.   It is re-
grettable that a complete list of Kossel's students is
not available at this time.

Kossel's ideas form the basis of many of our most
important biochemical concepts: "Kossel considered
the complex chemical constituents of the cell to be
formed from simpler compounds which he termed

Bausteine. These primary units were present in all living cells and from them were built by the processes of metabolism, the secondary building stones which vary from organism to organism. These ideas were placed on a sounder basis when at Kossel's motivation, Tamura carried out an analysis of bacterial cells and found that these small beings contained all the normal protoplasmic Bausteine."[29] In 1910, he received the Nobel Prize in Medicine for his work on the nucleus of the cell. He visited the U. S. in 1911 and his views of that time are summarized in his Herter lecture, "The Proteins",[37] and in his Harvey lecture "The Chemical Composition of the Cell."[36]

The story of the nucleic acids during the period from 1869-1909 is largely an account of the activities of Miescher and of Kossel and their students. Some of the milestones will be briefly outlined below. One of the points of interest is that while free purines had been found in nature, pyrimidines were unknown at the time the nucleic acids were discovered. The pyrimidines could not be isolated and characterized until the hydrolysis of the nucleic acids had been carried out.

C. *Outline of early landmarks in nucleic acid chemistry (1869-1909)* [6]

1869: Discovery of nucleic acids by Miescher. [15]
1874: Discovery of purine bases in nucleic acid. Piccard treated salmon sperm with boiling HCl, obtained guanine (unchanged) and hypoxanthine (derived from adenine). [51]
1879-80: Kossel hydrolysed nuclein from yeast, obtained xanthine and hypoxanthine. [32]
1885: Schultze and Bosshard isolated the first nucleoside, later shown to be guanosine. [56]
1885-86: Kossel hydrolysed nuclein from beef pancreas, obtained adenine, [33] converted it to hypoxanthine with nitrous acid. [34]

1889:   Altmann prepared and named nucleic acid, [1]
        protein-free, starting with yeast.
1891:   Kossel found carbohydrate liberated from
        yeast nucleic acid by acid hydrolysis. [35]
1894:   Hammarsten identified the carbohydrate as
        pentose. [25]
1894:   Kossel and Neumann prepared thymus nucleic
        acid from thymus gland and isolated thy-
        mine. [38]
1900-01: Ascoli in Kossel's laboratory, isolated
        uracil from yeast nucleic acid. [2]
1902-03: Kossel and Steudel, [39] and also Levene[41]
        isolated cytosine from thymus nucleic acid.
1909:   Levene and Jacobs identified the carbohy-
        drate of yeast nucleic acid as D-ribose, a
        previously unknown pentose. [43]
1909:   Levene and Jacobs isolated and identified
        adenosine. [44]

With the isolation and identification of D-ribose from
yeast nucleic acid, and the isolation of all of the pur-
ine and pyrimidine bases that were to be accepted as
nucleic acid components for the next fifty years, it
seems logical to consider this point as the end of an
era in the history of the nucleic acids. It was known
that the nucleic acids were built from simpler units
containing purines, pyrimidines, carbohydrate and
phosphate, but many years of difficult research were
to elapse before the presently accepted structure of
the nucleic acids could be proposed. Miescher had
been dead since 1895, and Kossel's interest shifted
to the chemistry of the basic proteins of the nucleus
after about 1905. The coming of World War I also
delayed progress in the nucleic acid field. The great
conflict brought a temporary end to the era of Inter-
national scholarship in the German laboratories. [29]
The new era of nucleic acid chemistry lay in other
hands and confusion reigned until about 1930 when
the steps toward progress were undoubtedly again
slowed by world wide depression and World War II.

The pent-up forces of fundamental research broke
out after the close of the second world war and the
phase of logarithmic growth began. We will now
attempt to chronicle some of the highlights in these
two periods.

III. THE PERIOD OF UNCERTAINTY (1909-1944)

A. *The old distinction between plant and animal nucleic acids versus the present interpretation*

Most of the early work on nucleic acids was carried
out with thymus nucleic acid, which yielded thymine,
and with yeast nucleic acid, which yielded uracil,
while adenine, guanine, and cytosine were common
to both. The characterization of the respective car-
bohydrate moieties was complicated by the fact that
thymus nucleic acid yielded levulinic acid, a com-
pound also obtainable from hexoses under similar
conditions of acid hydrolysis. Thus it was assumed
that the carbohydrate of thymus nucleic acid was a
hexose, and deoxyribose was not discovered until
enzymatic hydrolysis was employed. Since nucleic
acids of animal origin seemed to resemble thymus
nucleic acid, and the nucleic acid from wheat em-
bryo was similar to that from yeast, it was assumed
that the nucleic acids could be classified in a way
that correlated the hydrolysis products with the
source; the following Table 1.1, which is modified
from a standard 1934 textbook shows the status at
that time, with added comments in parenthesis.

However in a footnote it was stated that "the distinc-
tion between plant and animal nucleic acids is prob-
ably not as sharply drawn as has been supposed."
Various examples of isolation of "plant" nucleic
acids from animal sources and vice versa began to
appear but it was still stated in 1934 that "Nucleic
acids are present in the nuclei of cells. It is not

Table 1.1.   PRODUCTS OF HYDROLYSIS OF NUCLEIC ACIDS (1934)

of Animal Origin (i. e. thymis)	of Plant Origin (i. e. yeast, wheat embryo)
Phosphoric acid	Phosphoric acid
Adenine	Adenine
Guanine	Guanine
Cytosine	Cytosine
Thymine	Uracil
Levulinic and formic acids (under improved conditions D-2-deoxyribose is found)	Pentose (now D-ribose)

known with certainty that they ever occur in the cytoplasm." Although the presence of pentose nucleic acid in cytoplasm was suspected as early as 1905[5] it was not realized until the histochemical test of Feulgen[22] that the thymus or animal type occurred widely in the nuclei of both plants and animals. During this period scores of papers from Levene's laboratory were concerned with methods for hydrolyzing the nucleic acids both chemically and enzymatically, with the characterization of the split products, and with deductions as to the structure of the parent molecules. Since each paper was followed by additional papers with modifications of the previous methods or viewpoints, it is difficult to pinpoint the actual progress, and the student is urged to note the volume and titles of these papers under Levene's name in the author index of the Journal of Biological Chemistry to obtain a closer view of the trend during this period. The flood of papers by Levene on all subjects reached its peak in 1922-27 and 1928-33 when those in the Journal of Biological Chemistry alone averaged 23 publications a year for ten years.

Fortunately, summarizations for this period are available[21, 30, 42] but they are mainly of historical interest and the beginning student would do well not to read them until later. The identification of the carbohydrate moiety of the thymus type of nucleic acid depended on the preparation of purified nucleosides, which was accomplished enzymatically.[45] By mild acid hydrolysis the sugar was liberated and characterized as D-2-deoxyribose.[46]

The present position is that there are two main types of nucleic acid, deoxyribonucleic (DNA) and ribonucleic (RNA) with the following constituents, sources, and localizations:

Table 1.2.   MAJOR CONSTITUENTS OF NUCLEIC ACIDS

	DNA	RNA
Purines	Adenine and guanine	Adenine and guanine
Pyrimidines	Cytosine and thymine	Cytosine and uracil
Sugar	D-2-deoxyribose	D-ribose
Phosphate	One per sugar	One per sugar
Source	Plant and animal nuclei	Plant and animal cytoplasm and in nuclei
Former identification	Thymus, or animal nuclei acid	Yeast, or plant nuclei acid

The above conclusions were further buttressed by the histochemical studies by Brachet beginning in about 1933[7] and recently summarized[8], in which ribonuclease was used to specifically solubilize the ribonucleic acid in cells, and by the spectrophotometric studies of Caspersson beginning in about 1936[11] and recently summarized.[12]

The histochemical studies were supported by studies during 1942-46 in which the method of cell fractionation was employed. It was shown by Schneider[55] that isolated cell nuclei contained RNA and DNA while cytoplasmic fractions contained only RNA.

B. *The old tetranucleotide theory versus the present interpretation*

After the components of the nucleic acids were accounted for, the problem of their mode of linkage became acute, and much effort was devoted to its solution. The basic unit was the mononucleotide, which consisted of a nitrogenous base (purine or pyrimidine) attached to a pentose which in turn was attached to a phosphate. Since the analytical data of the 1930's indicated roughly equivalent amounts of the four bases, it was proposed that the nucleic acids consisted of a repeating "tetranucleotide" in which each base was represented once. Although the theory was never backed by analytical evidence, the lack of contrary evidence caused the idea to become very widespread in textbooks and at the time arbitrarily selected as the end of this period (1944) the tetranucleotide theory reigned supreme. Later, thanks to improved methods of separation and analysis, it was shown to be untenable and the smallest sub-unit with a repeating structure is now considered to be a mononucleotide.

Similarly, the mode of linkage between the mononucleotides was understood to involve a phosphate in diester linkage with each phosphate connected to two different pentoses, but the exact positions could not be established on the basis of the available evidence.

The resolution of these problems required new methods and new motivations that were set off by a startling report in 1944, which initiated what we will call the modern era.

IV. THE MODERN ERA OF STRUCTURE AND FUNCTION (1944-present)

A. A genetic function for DNA

It is here argued that the modern era of nucleic acid biochemistry was ushered in quite suddenly and unexpectedly by the report by Avery and coworkers[3] that DNA extracted from one type of bacteria could be taken up by another type which thereby was transformed to a new genetic constitution. The idea was strongly resisted on the basis that the DNA might be contaminated with protein, but this argument is no longer as compelling as it once seemed.

From a historical standpoint it appears that protein was in a preferred position for a genetic role prior to 1944. As early as 1911, Kossel[36] had expressed the view in these words, "The number of Bausteine which may take part in the formation of the proteins is about as large as the number of letters in the alphabet. When we realize that through the combination of letters an infinitely large number of thoughts can be expressed, we can understand how vast a number of properties of the organism may be re-corded in the small space which is occupied by the protein molecule." (italics mine). It does not appear that similar thoughts had been expressed regarding the nucleic acids. Although it was understood that chromosomes contained nucleoprotein, it was apparently never stated by anyone that the type of finding reported by Avery et al could be reasonably expected, or that nucleic acids alone might be able to carry all of the essential information of the heredity apparatus. Thus this report on the transforming ability of DNA raised theoretical questions about the "information-carrying capacity" of nucleic acids under the old tetranucleotide theory and provided added impetus to the demand for an understanding of nucleic acid structure. [16, 24]

B. *Improved techniques*

The modern era of nucleic acid chemistry could never have been developed if investigators had been limited to the methods used by Jones and Levene. This has been emphasized indirectly by Chargaff[14] in the following words: "The existence of significant biochemical differences between deoxypentose nucleic acids of different cellular origin, or what has been called the chemical specificity of nucleic acids, was discovered only a few years ago. [16] Soon after, it was possible to recognize most of the regularities that are recognized today. The steps that permitted this relatively very rapid progress were the following. After the development of partition chromatography on filter paper and its qualitiative application to amino acids it became obvious that the high and specific absorption in the ultraviolet of the purines and pyrimidines could form the basis of a quantitative ultra-micromethod, if proper procedures for the hydrolysis of the nucleic acids and for the complete separation of the hydrolysis products could be found. Such procedures were indeed developed." At about the same time, following the end of World War II, C^{14} - labeled metabolic precursors of the nucleic acids became widely available, and the combination of tracer techniques with the improved methods of separation set off an avalanche of data that has not begun to subside.

In this section some of the landmarks in the technical advance will be outlined.

1945: Schmidt and Thannhauser separated RNA and DNA for analytical purposes on the basis of the relative ease of hydrolysis of RNA by dilute alkali. [40, 53]

1945: Schneider separated nucleic acids from proteins by means of hot trichloroacetic acid.[40, 55]

1947: Vischer and Chargaff separated nucleic acid components by paper chromatography. [60]

1949:   W. Cohn separated nucleic acid components
        by means of ion exchange chromatography.[17]
1954:   Hurlbert, Schmitz, Potter and Brumm used
        extended gradient elution as a method for
        separating complex unknown mixtures of nu-
        cleotides by automatically changing the eluent
        concentration continuously over a wide range.[26]

C. *Modern landmarks*

The modern era of nucleic acid chemistry differs
from that of the Levene era in that the new emphasis
is on function and all of the chemical investigations
are closely intertwined with questions as to the func-
tion of the nucleic acids, as will be emphasized in
the later chapters of this book. From the historical
viewpoint, these contemporary results may easily
be misjudged. Nevertheless, we will attempt to out-
line some of the reports that seem to be landmarks
for today's students to remember, and to select
them on a yearly basis since the new methods have
become available.

1950:   Chargaff noted regularities in the ratios of
        adenine to thymine and guanine to cytosine in
        a variety of DNA preparations.[13]
1951:   Cohn and Volkin obtained 5' nucleotides from
        RNA by enzymatic hydrolysis.[18]
1952:   Markham and Smith deduced RNA structures
        from ribonuclease studies.[47]
1953:   Watson and Crick proposed a theory of DNA
        structure in terms of a double helix.[61]
1954:   Schmitz, Hurlbert and Potter isolated acid
        soluble polyphosphate ribonucleotides of all
        of the RNA components from animal tissues.[54]
1955:   Grunberg-Manago and Ochoa obtained RNA
        synthesis from riboside diphosphates by a
        soluble bacterial enzyme.[50]
1956:   Kornberg obtained DNA synthesis from deox-
        yriboside triphosphates by a soluble bacterial
        enzyme.[31]

1957:   Ingram found a single amino acid replaced
        in mutant hemoglobin. [28]
1958:   Schweet, Lamfrom and Allen showed protein
        synthesis in a cell-free system, integrating
        earlier studies on amino acid activation, and
        amino acid transport on soluble RNA. [57]

The above list inevitably omits many important
papers, but from the standpoint of the student it may
be worthwhile to note the chronology of the recent
landmarks and to relate contemporary advances to
them.   The chapters to follow will now consider the
various subdivisions of the subject in detail.

At this point it may suffice to say that as of 1958,
the outlines of the chemical basis for the role of
nucleic acids in genetics and physiology could be at
least roughly outlined. [52]   For a number of years to
come we can expect to witness a period of critical
examination and revision of some of the oversimpli-
fications that may have resulted from the rapid pace
of both theory and experiment in the period from
1944 to 1958.   In addition we may expect great ad-
vances in studies on the factors that modify gene
action, especially the phenomena of enzyme induc-
tion and the suppression of enzyme formation by
processes of negative feedback. [58]

REFERENCES

1.   ALTMANN, Arch. Anat. Physiol., physiol. Abteilung.
       p. 524 (1889).
2.   ASCOLI, Zeit. physiol. Chem. 31:161 (1900-01).
3.   AVERY, MacLEOD and McCARTY, J. Exp. Med. 79:
       137 (1944).
4.   BARNETT, A Century of Darwin, Heinemann, London
       (1958).
5.   BEEBE and SHAFFER, Am. J. Physiol. 14:231 (1905).
6.   BENDICH, in Chargaff and Davidson, 1:81 (1954).

7.   *BRACHET, Arch. Biol. (Liege) 44:519 (1933).*
8.   *BRACHET, Biochemical Cytology, Academic Press,*
      New York, 1957.
9.   *BROWN, The New Physician 7:No. 10:15 (1958).*
10.   *CARTER, Nature 182:1351 (1958).*
11.   *CASPERSSON, Skand. Arch. Physiol. 74:Supp. 8 (1936).*
12.   *CASPERSSON, Cell Growth and Cell Function, W. W.*
      Norton & Co. New York (1950).
13.   *CHARGAFF, Experientia 6:201 (1950).*
14.   *CHARGAFF, in Chargaff and Davidson 1:307 (1954).*
15.   *CHARGAFF and DAVIDSON, in Chargaff and Davidson*
      1:1 (1954).
16.   *CHARGAFF, VISCHER, DONIGER, GREEN and*
      MISANI, J. Biol. Chem. 177:405 (1949).
17.   *COHN, W., Science 109:377 (1949), J. Am. Chem.*
      Soc. 72:1471 (1950).
18.   *COHN and VOLKIN, Nature 167:483 (1951).*
19.   *de BEER, Endeavour 17:61 (1958).*
20.   *ELVEHJEM, Nat. Acad. Sci. Biog. Mem. 28:No. 5*
      (1954).
21.   *FEULGEN, Chemie und Physiologie der Nukleinstoffe,*
      Borntraeger, Berlin (1923).
22.   *FEULGEN and ROSSENBECK, Zeit. physiol. Chem.*
      135:203 (1924).
23.   *GILLESPIE, Am. Sci. 46:388 (1958).*
24.   *GULLAND, Cold Spring Harbor Symp. 12:95 (1947),*
      Symp. Soc. Exp. Biol. 1:1 (1947).
25.   *HAMMARSTEN, Zeit. physiol. Chem. 19:19 (1894).*
26.   *HURLBERT, SCHMITZ, POTTER and BRUMM, J.*
      Biol. Chem. 209:23 (1954).
27.   *HUXLEY, J. Linn. Soc. London 44:1 (1958).*
28.   *INGRAM, Nature 180:326 (1957), B. & B. Acta. 28:539*
      (1958).
29.   *JONES, Mary Ellen, Yale J. Biol. Med. 26:80 (1953).*
30.   *JONES, W., Nucleic Acids 2nd Ed. Longmans Green*
      & Co. London (1924).
31.   *KORNBERG in McElroy and Glass, The Chemistry of*
      Heredity. Baltimore, 1957 and LEHMAN, BESSMAN,
      SIMMS and KORNBERG J. Biol. Chem. 233:163,
      171 (1958).

32. KOSSEL, *Zeit. physiol. Chem.* 3:284 (1879), 4:290 (1880).
33. KOSSEL, *Ber.* 18:79 (1885), *Zeit. physiol. Chem.* 10:248 (1886).
34. KOSSEL, *Ber.* 18:1928 (1885).
35. KOSSEL, *Arch. Anat. Physiol.*, *physiol. Abteilung* p. 18 (1891).
36. KOSSEL, *Harvey Lectures, Philadelphia* 7:33 (1911-12).
37. KOSSEL, *Bull. Johns Hopkins Hosp.* 23:65 (1912).
38. KOSSEL *and* NEUMANN, *Ber.* 27:2215 (1894).
39. KOSSEL *and* STEUDEL, *Zeit. physiol. Chem.* 37:177 (1902-03).
40. LESLIE, *in Chargaff and Davidson* 2:1 (1954).
41. LEVENE, *Zeit. physiol. Chem.* 37:402 (1902-03).
42. LEVENE *and* BASS, *Nucleic Acids, Chem. Catalog Co. New York* (1931).
43. LEVENE *and* JACOBS, *Ber.* 41:2703 (1908); 42:1198 (1909); 44:746 (1911).
44. LEVENE *and* JACOBS, *Ber.* 42:2703 (1909).
45. LEVENE *and* LONDON, *J. Biol. Chem.* 81:711 (1929); 44:746 (1911).
46. LEVENE, MIKESKA *and* MORI, *J. Biol. Chem.* 85:785 (1930).
47. MARKHAM *and* SMITH, *Biochem. J.* 52:552,558,565 (1952).
48. MATTHEWS, *J. Linn. Soc. London* 44:93 (1958).
49. NORDENSKIOLD, *The History of Biology, Tudor, New York* (1935).
50. OCHOA, *Fed. Proc.* 15:832 (1956).
51. PICCARD, *Ber.* 7:1714 (1874).
52. POTTER, *Fed. Proc.* 17:691 (1958).
53. SCHMIDT *and* THANNHAUSER, *J. Biol. Chem.* 161:83 (1945).
54. SCHMITZ, HURLBERT *and* POTTER, *J. Biol. Chem.* 209:43 (1954).
55. SCHNEIDER, *J. Biol. Chem.* 161:293 (1945).
56. SCHULTZE *and* BOSSHARD, *Zeit. physiol. Chem.* 9:420 (1885), 10:80 (1885).
57. SCHWEET, LAMFROM, *and* ALLEN, *Proc. Nat. Acac. Sci. U. S.* 44:1029 (1958).

58. UMBARGER and BROWN, J. Biol. Chem. 233:415
      (1958).
59. VICKERY and SCHMIDT, Chem. Rev. 9:169 (1931).
60. VISCHER and CHARGAFF, J. Biol. Chem. 168:781
      (1947).
61. WATSON and CRICK, Nature 171:737, 964 (1953),
      Cold Spring Harbor Symp. 18:123 (1953).

Chapter II
Structure of Bases,
Nucleosides and Nucleotides

I. THE PURINE BASES

    A.   Major purines occurring in the nucleic acids
    B.   Trace purines occurring in nucleic acids
    C.   Purine analogs occurring in nucleic acids
    D.   Purines not found in nucleic acids

II. THE PYRIMIDINE BASES

    A.   Major pyrimidines occurring in nucleic acids
    B.   Pyrimidine analogs occurring in nucleic acids
    C.   Pyrimidines not found in nucleic acids

III. THE STRUCTURE OF THE NUCLEOSIDES

    A.   Generalization on the structure of the nucleosides obtained from nucleic acids
    B.   Names of the nucleosides
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IV. THE STRUCTURE AND ROLE OF THE NUCLEO-
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    A.   Definition and classification of the nucleotides
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    C.   Names of the nucleotides
    D.   Early work with 3' and 5' nucleotides
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    G.   The acid soluble 5' nucleotides
    H.   The role of the nucleotides in metabolic pathways

Chapter II
Structure of Bases,
Nucleosides and Nucleotides

I. THE PURINE BASES

  A. Major purines occurring in nucleic acids

There are two purines that occur in both RNA and
DNA almost to the exclusion of all others. These
are <u>adenine</u> and <u>guanine</u>, and their predominance is
so overwhelming that they were discovered in the
first days of nucleic acid investigations (1874-1885),
while no other purines were found in the nucleic
acids until 1955, and then only in traces. Since all
nucleic acid preparations are now assumed to be
mixtures of unique sequences of nucleotides, the
characteristic amount of any given base is merely
a statistical average of all the nucleic acid mole-
cules in the preparation. In the ox, sheep, pig, or
man, the ratio of adenine to guanine in DNA prepa-
rations is about 3:2. [12, p. 369]   The same ratio
appears to obtain with yeast and several microor-
ganisms while in other cases the ratio may be re-
versed or may approach 1:1, but in general, does
not exceed the limits mentioned. [12, p. 359] The struc-
tures of these two important bases are given below,
with the purine structure numbered for reference.

Adenine =   Guanine =

Purine   6-amino-purine   2-amino-6-oxy-purine
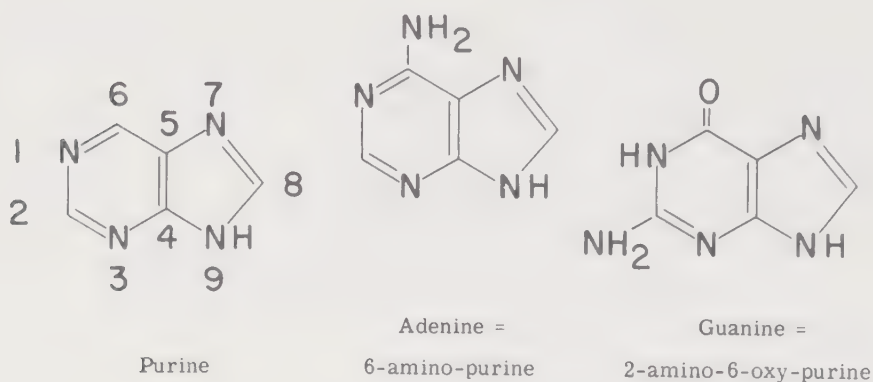
Fig. 2.1.   Structure and numbering of the major purines in nucleic acids.   The
oxygen in guanine is shown in the keto form.

B.   *Trace purines occurring in nucleic acids*

1.   In 1955 Dunn and Smith[21] reported the occur-
rence of a purine other than adenine or guanine
in a nucleic acid, and since that time further
reports have appeared.[1, 31]   The new purine
has been identified as 6-methyl-amino purine,
i.e., adenine with a methyl substitution on the
amino group.   The compound was first found in
DNA from the thymine-requiring E. coli mutant
15T⁻, and appeared when the bacteria were
grown with the thymine concentration at low
levels or in the presence of the thymine anal-
ogues 5-aminouracil or 2-thiothymine.   It ap-
peared to replace thymine to a limited extent
and amounts as high as 4.2 moles per 100
moles of total base could be attained in prep-
arations in which the thymine content was 19.1
moles per 100 moles total base.

2.   In 1958 Littlefield and Dunn[31] reported addi-
tional trace purines at levels below one mole
per 100 moles of base in RNA from E. coli
B/r and 15T⁻, Aerobacter aerogenes, yeast

and rat liver microsomes. The new compounds were 2-methyl-adenine and 6-dimethyl-amino-purine. In addition, 6-methyl-amino purine was found in RNA from these sources.

3. The above compounds were all derivatives of adenine. Trace guanine derivatives in yeast RNA were reported by Adler et al.[1] N^2-methyl guanine and 1-methyl guanine were reported in addition to 6-methyl-amino-purine reported earlier. It must be pointed out that although these bases are present in trace amounts, the usual preparations of nucleic acids contain so many "species" of nucleic acid that the findings may be due to the presence of the new bases in only a few of the species. At this time nothing more can be said about their significance. The structures of the trace purines are shown in Fig. 2.2.

6-methyl-amino-purine

6-dimethyl-amino-purine

2-methyl-adenine

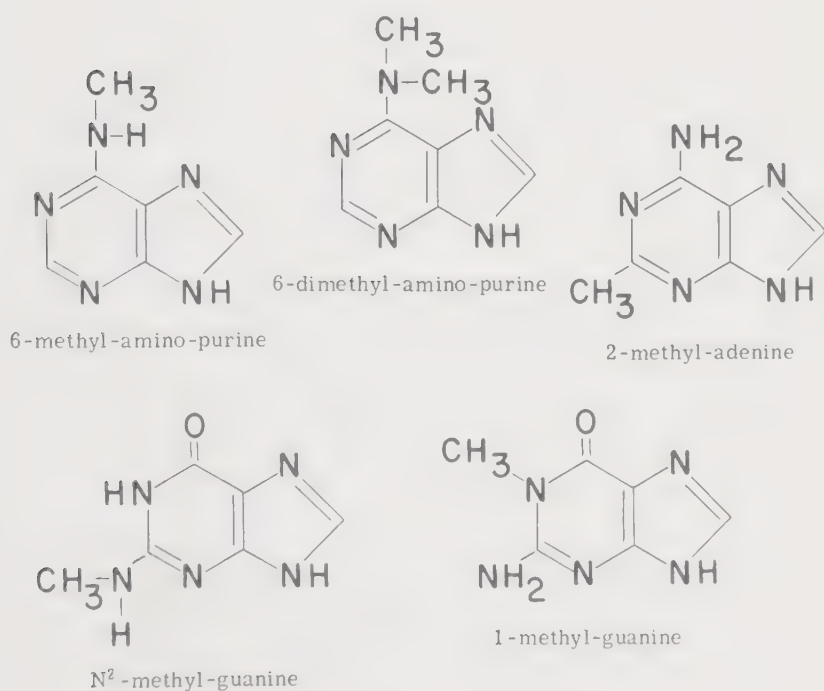N^2-methyl-guanine

1-methyl-guanine

Fig. 2.2. Structures of some trace purines from nucleic acids

C. *Purine analogs occurring in nucleic acids*

The new purines referred to above were natural components of the nucleic acids. With the advent of purine anti-metabolites such as 2,6-diaminopurine or 8-azaguanine, several of the unnatural purines have been shown to be actually incorporated into the nucleic acids to give what are assumed to be "fraudulent" nucleic acids with altered specificities. These will be discussed in Chapter XVIII.

D. *Purines not found in nucleic acids*

There are a number of purines occurring free or in nucleoside form in nature.[5] These include the catabolic products hypoxanthine, xanthine, and uric acid (Chapter VIII) and the alkaloids caffeine, theobromine and theophylline, which are, respectively, 1:3:7 trimethylxanthine, 3:7 dimethylxanthine, and 1:3 dimethylxanthine, and are found in coffee, cocoa and tea.[13]

II. *THE PYRIMIDINE BASES*

A. *Major pyrimidines occurring in nucleic acids*

The pyrimidines are structurally closely related to benzene, pyrimidine and the triazines, as Pinner pointed out in 1885. These structures are shown in Fig. 2.3 with the two pyrimidine numbering systems in current use. The present Outlines will employ the Chemical Abstracts numbering system.

As in so many examples in chemistry, the pyrimidines can be grouped into a kind of periodic table in which a basic structure appears with a number of possible variations. In the case of the purines, positions 2 and 6 are responsible for specificity, occurring in nucleic acids with H, O, or NH_2 as substituents. In the case of the pyrimidines, the significant positions are 4 and 5, with the 2 posi-
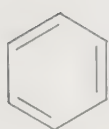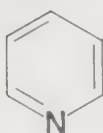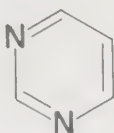
Benzene   Pyridine   Pyrimidine   s-Triazine   Asymmetrical
                                                                       triazine, or
                                                                       6-aza-pyrimidine
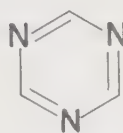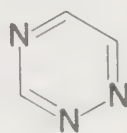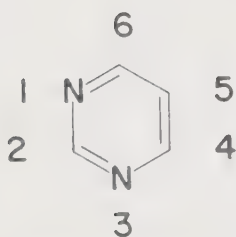
Older numbering system   Chemical Abstracts system
(parallels purine system)
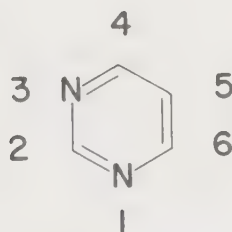
Fig. 2.3.   Structure and numbering of pyrimidines and comparison with related
            compounds. Note that the 2 and 5 positions have the same relation to
            the nitrogens in both numbering systems. The Chemical Abstracts sys-
            tem will be used in the material to follow.

tion as a hydroxy derivative in all cases. As in
the case of guanine, the hydroxy derivative is
mainly present in the keto form. The major
pyrimidines found in the nucleic acids may accord-
ingly be described in systematic terms as follows:

Table 2.1.   PYRIMIDINE SUBSTITUENTS ARRANGED SYSTEMATICALLY

	H on carbon 5	CH_3 on carbon 5
Oxygen on carbon 4	uracil	thymine (i.e., 5-methyl uracil)
NH_2 " " "	cytosine	5-methyl cytosine
These structures are shown in Fig. 2.4.		
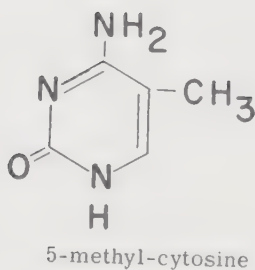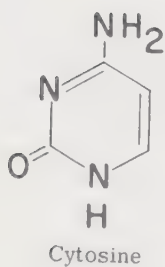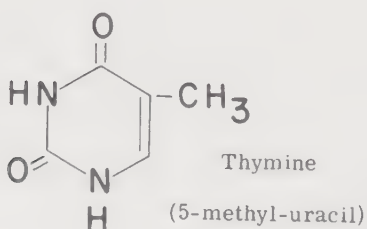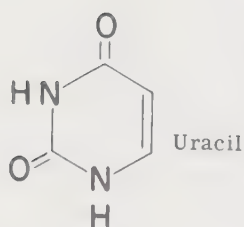
Fig. 2.4.   Systematic arrangement of the major pyrimidines found in nucleic acids. Certain bacterial viruses contain 5-hydroxymethyl-cytosine in place of cytosine.

Until 1950 only three of the pyrimidines shown in
Fig. 2.4 had been discovered. These were thymine
(1894), uracil (1900), and cytosine (1902). By 1925
it was thoroughly established that thymine was pe-
culiar to DNA, while uracil was found only in RNA,
and cytosine was found in both RNA and DNA from a
variety of sources. The analogies between the
pairs of compounds illustrated in Figure 2.4 led
biochemists to suspect the existence of 5-methyl-
cytosine for many years, and its presence in a
nucleic acid from tubercle bacilli was announced in
1925 but is now doubted.[5]

1. The discovery of 5-methyl cytosine in DNA
    (1950) and RNA (1958)
    With the background indicated above, and the
    availability of new methods (paper chroma-
    tography) for detecting trace components of
    nucleic acids, it was not surprising that 5-
    methyl cytosine should be found, but many
    features of its distribution could not be antic-
    ipated. A thorough search by Wyatt[43] revealed
    the compound in trace amounts in DNA from
    mammalian, fish, and insect sources and in
    much higher amounts in wheat germ DNA, but
    absent from bacteria including tubercle bacilli,
    viral sources, and until recently, from RNA.
    In wheat germ DNA, the 5-methyl cytosine
    appears to replace cytosine on a 1:1 basis to
    the extent of about 25% of the total cytosine
    that might be predicted from the guanine-cyto-
    sine pairing.[1, p. 358]
       In 1958 Littlefield and Dunn[31] reported the
    occurrence of thymine in several bacterial
    RNA preparations in trace amounts. This was
    followed by a study of another strain of E. coli
    in which Amos and Korn[2] reported the occur-
    rence of trace amounts of 5-methyl cytosine in
    the RNA, and suggested that this compound
    would have been converted to thymine by the
    conditions used by Littlefield and Dunn.

However their criticism would not apply to the
enzymatic studies by the Cambridge workers.

2.   The discovery of 5-hydroxymethyl cytosine and
      derivatives in DNA (1952)
      In 1951 Marshak[32] reported the absence of cyto-
      sine from the DNA of a bacterial virus, the
      coliphage T_2. The studies referred to in the
      previous section pointed to the possible sub-
      stitution of 5-methyl cytosine for cytosine in
      this preparation but it was soon established by
      Wyatt and Cohen[44] that the cytosine was not
      replaced by 5-methyl cytosine but by a new
      pyrimidine, 5-hydroxymethyl cytosine in the
      DNA from the T_2, T_4 and T_6 phages of E. coli.
      Thus far the new pyrimidine has not been found
      in any other source. The hope that a general
      explanation of viral properties might emerge
      soon faded when Sinsheimer[37] found that the T_7
      coliphage DNA did not contain 5-hydroxymethyl
      cytosine but contained cytosine. In all cases
      the host DNA contains cytosine but none of the
      5-hydroxymethyl cytosine.

      The unique aspects of DNA from the T-even
      coliphages did not end with the discovery of
      5-hydroxymethyl cytosine, however, for it was
      soon found by Sinsheimer[36] and by Volkin[41]
      that glucose residues were in glucoside linkage
      with the hydroxymethyl group. Further work
      by Jesaitis[27] and by Cohen[14] showed that the
      amount of glucose varied in a characteristic
      way with the strain of phage tested, and that
      in any given phage the 5-hydroxymethyl cyto-
      sine was not all combined with glucose.
      Nevertheless, the total amount was very nearly
      equal to the total amount of guanine on a molar
      basis, which is the relationship that holds for
      guanine and cytosine in DNA's that contain no
      5-methyl cytosine or its hydroxymethyl deriv-
      ative, and for guanine and the sum of cytosine
      and 5-methyl cytosine in DNA from wheat

germ[12], [p. 360]. The significance of these re-
lationships will be discussed in Chapter X.

3. The discovery of pseudo-uridine in RNA (1957)
    Cohn[18] and Davis and Allen[20] reported the
    presence of a new nucleotide in yeast RNA and
    Kemp and Allen[28] found the "fifth" nucleotide
    in pancreas RNA. Further work by Cohn in-
    dicates that the compound is uracil-5-ribityl
    and again calls attention to the variation that
    is possible in the 5 position of the pyrimidine
    ring.

B. Pyrimidine analogs occurring in nucleic acids

As in the case of the purines, the development of
pyrimidine analogs such as bromo-uracil and
fluoro-uracil has been followed by the discovery
that these compounds can in some cases be incor-
porated into RNA or DNA to give products not
found in nature. These compounds will also be dis-
cussed in Chapter XVII.

C. Pyrimidines not found in nucleic acids

In addition to the pyrimidines that have been ob-
tained from nucleic acids, the pyrimidine nucleus
has been found in nature in the form of derivatives
that so far have not been obtained from the nucleic
acids. A series of compounds known as vicines
was isolated as early as 1870 from the vicia leg-
umes, beet juice and peas. Divicine is 2:6-diamino-
4:5 dihydroxy pyrimidine while vicine has a D
glucose residue on the 5 hydroxy group. Convicine
is believed to be a glycoside of 5-hydroxy-6-amino
uracil[5]. While no function for these compounds is
established, another pyrimidine that once was a
curiosity is now a well established intermediate in
pyrimidine biosynthesis. This is uracil-6-car-
boxylic acid, usually referred to as orotic acid
from its original source which was whey (Greek:

oros). Its role will be described in Chapter VI.
It was first isolated in 1905[5]. Another important
pyrimidine derivative is vitamin B_1 which can be
thought of as a 5:6-dimethyl uracil with a thiazole
replacing one hydrogen of the 5-methyl group.

There are virtually no recent systematic data
available on the concentration of free purines or
pyrimidines in plant or animal tissues but the in-
dications are that the levels are extremely low.
In general, the free purines or pyrimidines are
either converted to higher forms, nucleosides or
nucleotides, or to lower forms that are completely
degraded.

III.   THE STRUCTURE OF THE NUCLEOSIDES

A.   Generalization on the structure of the nucleosides obtained from nucleic acids

"Nucleoside" is the name given to a special class
of carbohydrate derivatives in which the nitrogenous
bases found in nucleic acids (purines or pyrimidines)
are in glycosidic linkage with a pentose. Thus a
nucleoside = nucleic acid base + pentose sugar. In
the case of the "exotic" nucleosides, it has not been
established how much deviation from conventional
nucleoside structures can occur before the com-
pound is excluded from the nucleoside classification.
The generalizations to follow apply to the "normal"
nucleoside structure as found in the nucleosides ob-
tained from nucleic acids up to the present.

1.   Nature of sugars
     Thus far no sugars other than D-ribose (from
     RNA) and D-2-deoxyribose (from DNA) have
     been found in nucleoside linkage in nucleic
     acids. The occurrence of glucose not in nucleo-
     side linkage in DNA from T_{even} coliphage has
     been noted above, but in these instances, the
     nucleoside is apparently still a D-2-deoxyribo-
     side.

2.   Nature of linkages
      The linkages between base and sugar are the
      same for the deoxyriboses as for the ribosides.
      In both, carbon no. 1 of the pentose is always
      attached to nitrogen no. 9 of the purines and to
      nitrogen no. 1 of the pyrimidines, in β gluco-
      side configuration. (For a time the purine
      attachment was considered to be on nitrogen
      no. 7.) In the nucleoside the pentose numbers
      are 1', 2', 3' etc. to distinguish them from the
      numbered atoms in purine or pyrimidine.

On the basis of the above rules, and the listing of
the available purines and pyrimidines in sections I
and II the student should be able to draw the struc-
tures of each of the nucleosides that are known to
occur in nucleic acids. It will be helpful in pro-
gressing to the three-dimensional structure of the
nucleic acids if the nucleosides are visualized in
the configuration in which they are believed to occur
in the nucleic acids. Two examples will be given,
and all the other nucleosides can be written in
analogous forms according to the four generaliza-
tions given. See Fig. 2.5.
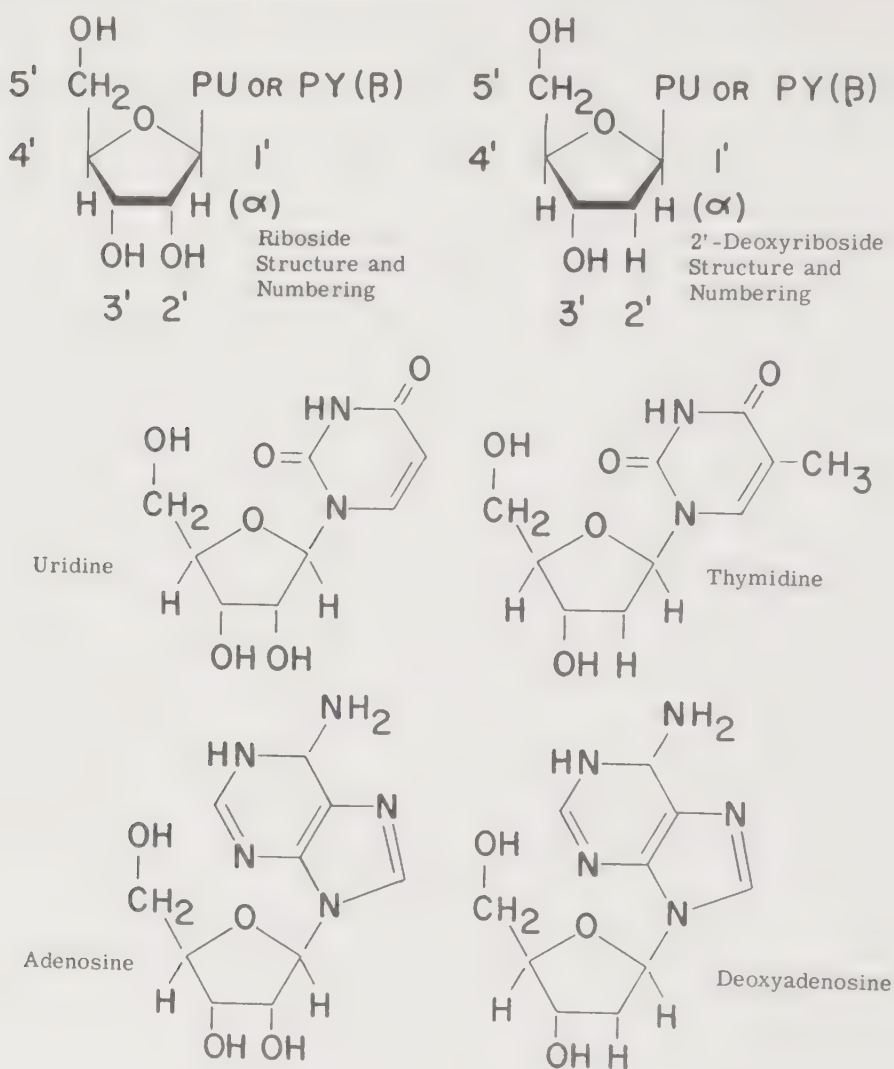
Fig. 2.5. The structure of some representative nucleosides. The upper structures
show the numbering system for a β-D-riboside and a β-D-2'-deoxy-
riboside and indicate the β configuration on the 1' carbon of the
pentoses. The middle structures are uridine, or 1-β-D-ribofurano-
syluracil, and thymidine, or 1-β-D-2'-deoxyribofuranosylthymine.
The lower structures are adenosine, or 9-β-D-ribofuranosyladenine,
and deoxyadenosine, or 9-β-D-2'-deoxyribofuranosyladenine. Other
nucleoside structures can be obtained by substituting the appropriate
bases for the ones shown here. PU=purine base; PY=pyrimidine base.

B. *Names of the nucleosides*

The following names are in general use for the more common nucleosides.

Table 2.2. NAMES OF NUCLEOSIDES

Base	Riboside	Deoxyriboside
adenine	adenosine	deoxyadenosine
guanine	guanosine	deoxyguanosine
uracil	uridine	deoxyuridine
cytosine	cytidine	deoxycytidine
thymine	thymine riboside*	thymidine

* Nomenclature not established; has been called ribothymidine also.

All of the nucleosides listed above are known to occur in animal tissues or in extracts of animal tissues under particular conditions, and they probably occur in plants and bacteria as well. The roles of deoxyuridine and of thymine riboside are as yet not understood, although deoxyuridine may be related to thymidine biosynthesis (Chapter VI). In all cases the levels of the nucleosides are very low and in dynamic equilibrium with the nucleotides and the bases.

C. *Exotic nucleosides*

We propose the name exotic nucleosides for certain compounds found in nature in the free form and not in nucleic acids, nor as known nucleic acid precursors or degradation products. Bendich[5] has mentioned nebularine, a "normal" riboside of purine, and cordycepin, puromycin, and spongosine, all of which contain unusual sugars, bases or both. Several of the compounds are antibiotics.

*IV.   THE STRUCTURE AND ROLE OF THE NUCLEO-
        TIDES*

A.   Definition and classification of the nucleotides

By definition, a nucleotide is a phosphate ester of
a nucleoside. In general, the word nucleotide is
used to mean mono-nucleotide, while di-, tri-, and
polynucleotides are so specified. A mononucleo-
tide consists of one nucleoside (base and sugar)
and one or more phosphates, which may carry addi-
tional substituents (see Chapter IX on Coenzymes).
An examination of the structure of a ribonucleoside
reveals that there are only 3 OH groups available
for esterification with phosphate, namely the 2',
3' and 5' positions. In the case of the deoxyribo-
sides, only the 3' and 5' positions are available.
It will be shown that all of the possible phosphate
esters are indeed known, and today it is necessary
to specify the position of the phosphate as 2', 3',
5' or 2', 3' cyclic when a nucleotide is mentioned.
These structures are shown in Fig. 2.6, using
uridine mono-phosphates as examples, with uri-
dine-5'-phosphate, thymidine-5'-phosphate and
thymidine-5'-triphosphate for comparison.
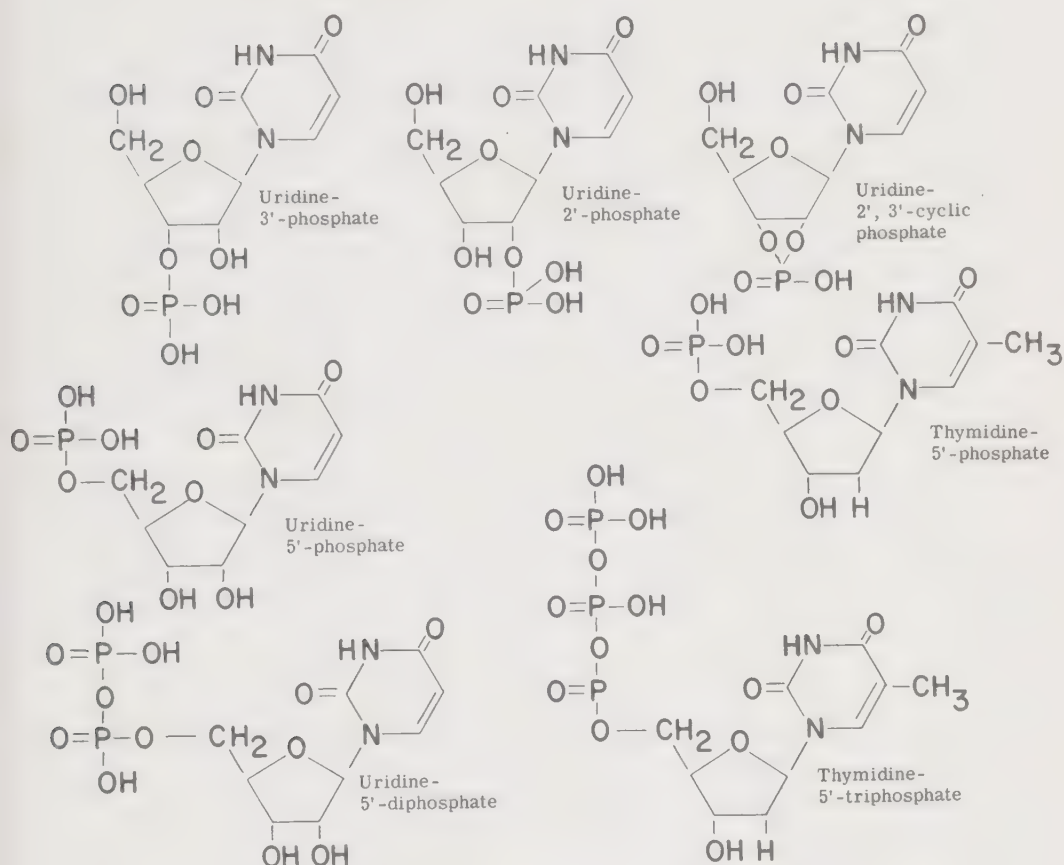
Fig. 2.6. The structure of some representative mononucleotides.. The 2', 3', 5', and 2', 3' cyclic monophosphates and the 5'-diphosphate of uridine are shown, and the 5'-phosphate and 5'triphosphate of thymidine are shown. These structural formulations may be compared with the nucleic acid structures shown in Chapter III. The remaining nucleotide structures can be obtained by substituting the appropriate bases for the ones shown here. The numbering of the pentose carbons is shown in Fig. 2.5. All hydrogens on rings have been omitted.

B. *Acid soluble nucleotides*

A word of explanation is needed in order to clarify the place of <u>acid-soluble</u> <u>nucleotides</u>, which were represented exclusively by the adenine nucleotides prior to the years 1950-54. In the first place, the term is an operational one and refers to the material occurring in the filtrate when the proteins are precipitated with cold trichloracetic acid or perchloric acid. These filtrates are considered to include only nucleotides not in polymeric form. The term should not be applied to filtrates obtained by the action of <u>hot</u> trichloroacetic acid by the technique of W. C. Schneider and in general has not been so applied.

Studies on the nucleotides of nucleic acids prior to the widespread use of isotopic tracer techniques tended to be concerned with the structure of the nucleic acids as deduced from their breakdown products, while the studies on the acid soluble nucleotides involved only their role as coenzymes. The lack of connection between the two lines of investigation lasted until recently at least in part because of the fact that the hydrolysis products of the nucleic acids included only the 3' nucleotides and not the 5' variety, while the acid soluble nucleotides included only 5' nucleotides and none of the 3' variety. Moreover, there was no evidence that the 5' nucleotides were convertible to the nucleic acids. The dichotomy between the 3' and the 5' nucleotides was ended during the years since 1950 when the 5', 2', 3', and 2', 3' cyclic phosphates of all the components of RNA were obtained. The present position is summarized at this point and will be discussed in sections D to H.

1. The question of whether 2', 3', 5' or 2' 3' cyclic phosphates are obtained from nucleic acids is a matter of what substrates, enzymes and conditions are used for the hydrolysis.

2. All of the 5' nucleotides obtainable from RNA exist in the acid soluble fraction of a variety of tissues.

3. The 5' nucleotides obtainable from DNA appear to be present in the acid soluble fraction of tissues actively synthesizing DNA.

4. Evidence for direct conversion of 5' nucleotides to RNA and DNA in extracts of bacteria and of animal tissues is available.

5. Evidence that the 5' nucleotides exist in metabolic pools convertible to either coenzymes or to nucleic acids is available.

6. At this writing there is no evidence indicating the presence of 3' nucleotides in the acid soluble fraction of biological material and no evidence for their occurrence as intermediary metabolites, although there is reason to assume that they occur as digestion products of nucleic acids and perhaps in autolyzing tissue.

C. *Names of the nucleotides*

There were in general two names in common usage for each of the mono-nucleotides that occur as a monophosphate. A given nucleotide could be called a monophosphate of the corresponding nucleoside, or it could be called an acid with a name derived from the corresponding nucleoside (rather than from the base). Now that the phosphate is known to occupy a variety of positions, it is necessary to specify the position. The resulting names are listed on the following page:

Table 2.3.   NAMES OF NUCLEOTIDES

Common Ribonucleotides		
Corresponding Base	Nucleotide Named as Acid	Nucleotide Named as Phosphate
Adenine	2' Adenylic acid or Adenylic acid a	Adenosine 2'-monophosphate
Adenine	3' Adenylic acid or Adenylic acid b	Adenosine 3'-monophosphate
Adenine	5' Adenylic acid	Adenosine 5'-monophosphate*
Adenine	Cyclic Adenylic acid	Adenosine 2', 3'-monophosphate (cyclic)
Guanine	2' Guanylic acid, etc. **	Guanosine 2'-monophosphate, etc.
Uracil	2' Uridylic acid, etc.	Uridine 2'-monophosphate, etc.
Cytosine	2' Cytidylic acid, etc.	Cytidine 2'-monophosphate, etc.
Hypoxanthine	2' Inosinic acid, etc.	Inosine 2'-monophosphate, etc.
Common Deoxyribonucleotides		
Corresponding Base	Nucleotide Named as Acid	Nucleotide Named as Phosphate
Adenine	Deoxyadenylic Acid	Deoxyadenosine 5'-monophosphate*
Guanine	Deoxyguanylic Acid	Deoxyguanosine 5'-monophosphate*
Thymine	Thymidylic Acid	Thymidine 5'-monophosphate*
Cytosine	Deoxycytidylic Acid	Deoxycytidine 5'-monophosphate*

* Each of the 5' monophosphates also occurs as the diphosphate and the triphosphate.
** Etc. indicates that each 2' compound occurs in other forms corresponding to the series shown for adenine.

D.   *Early work with 3' and 5' nucleotides* [3]

The first nucleotide to be discovered was 5'-inosinic acid or muscle inosinic acid, the 5' monophosphate of inosine. It was discovered in 1847 by Liebig, who isolated it from meat extract. It was undoubtedly an artefact arising from the deamination of 5'-adenylic acid. It was not

characterized at that time but was shown to contain the base hypoxanthine in 1895.

In 1918 Levene obtained all of the 3' nucleotides from RNA of yeast including 3' adenylic acid or yeast adenylic acid.

In 1927 Embden and Zimmerman obtained muscle adenylic which was shown to be a 5' adenylic acid. The relationship of this compound to muscle inosinic acid was shown by Gerhard Schmidt in 1928 when he prepared adenylic deaminase from muscle and converted the 5'-adenylic to 5'-inosinic acid. The enzyme was inactive towards yeast adenylic acid.

The above studies provided reference points for later investigators and set up the dichotomy of muscle adenylic and yeast adenylic that persisted until about 1951.

Thus when ATP was isolated in 1929, by Lohmann and by Fiske and Subbarow, it was shown to be convertible to "muscle" adenylic acid and was therefore the 5' variety.

In 1932 Levene and Harris obtained yeast xanthylic acid from yeast guanylic and in 1933 they obtained "yeast" inosinic acid (the 3') from yeast adenylic acid.

When the first deoxyribose monophosphates were obtained by Klein and Thannhauser in 1933 by treating DNA with intestinal extracts in the presence of arsenate they were assumed to be 3' because they came from nucleic acid.

When the adenine nucleotide coenzymes DPN, TPN, and FAD were discovered in 1934-1938 they were shown to be based on 5' adenylic acid.

By 1949 the distinction between "yeast" (or nucleic
acid) adenylic and "muscle" (or coenzyme) adenylic
had not broken down.   No 5' nucleotides other than
5' AMP and its derivatives were known in the free
form, and no 3' nucleotides were found in tissues,
and no fully characterized nucleotides from either
RNA or DNA had been obtained.   This is because
prior to this time it was not known that both the 2'
and 3' monophosphates were produced by nucleic
acid hydrolysis, nor was it known that these ribo-
nucleotides are readily interconvertible under mild
acid conditions.[3]

E.   *The resolution of 2' and 3' nucleotides*

In 1949 Waldo Cohn[15] introduced ion exchange
chromatography to the problem of separating the
hydrolysis products of the nucleic acids and soon
discovered[16] that each of the 4 nucleotides that
had formerly been assumed to be the 3' variety
appeared on the chromatogram in two places, and
were assigned provisional designations a and b.
Meanwhile Brown and Todd[8] had prepared the 2'
and 3' adenylic acids chemically and a series of
papers from both laboratories established the
identity of the a nucleotides with the 2' phosphates,
and the b nucleotides with the 3' phosphates.[9]
Owing to the systematic way in which the nucleo-
tides are eluted from the ion exchange columns,
the order in which the 2' and 3' nucleotides are
eluted is the same for each of the 4 nucleotides.
In each case the 2' phosphates are eluted ahead of
the 3' nucleotides, while the 5' nucleotides come
off ahead of both the 2' and the 3' varieties.[17]

F.   *The enzymatic hydrolysis of nucleic acids to yield*
      5'-nucleotides

In 1951, Cohn and Volkin[19] compared a variety of
methods for hydrolysing the nucleic acids and ex-
amined the products with the new methods of ion-

exchange chromatography. The 5' nucleotides were readily separated from the 2' and 3' variety and characterized by chemical properties and comparison with known 5' nucleotides. It was found that the 5' nucleotides could be obtained by treating RNA with a phosphodiesterase obtained from snake venom which had been purified sufficiently to remove the 5' phosphomonoesterase. These digests also contain cytidine and uridine 2', 5'- and 3' 5'-diphosphates, which will be discussed in Chapter III. Here it will be noted that the isolation of the 5' ribonucleotides from RNA and the 5' deoxyribonucleotides from DNA by Volkin, Khym and Cohn[42] paved the way for the complete breakdown of the artificial barriers formerly separating the 5' nucleotides from the nucleic acids.

G. *The acid soluble 5' nucleotides*

The first clue that the acid soluble fraction of cells contains nucleotides other than those of adenine came from the laboratory of Luis Leloir in 1950 when it was reported[10] that the coenzyme for the glucose-galactose transformation is uridine-5'-diphosphate glucose. Further indications came in 1952 when Park,[33] following earlier work by Park and Johnson, reported the occurrence of additional UDP derivatives in penicillin inhibited Staph. aureus. These reports alerted biochemists to the existence of uridine nucleotides in the acid soluble fraction but gave no indication that the acid soluble nucleotides were connected to nucleic acid synthesis.

The latter conclusion emerged from studies on the metabolism of orotic acid-6-C^{14} reported by Hurlbert and Potter in 1952-54, who with Busch, Schmitz and Brumm modified the ion exchange methods of Cohn specifically for the problem of dealing with the acid soluble fraction, which in the case of animals previously injected with orotic

acid-6-C^{14} was shown to contain a large number of previously unidentified nucleotides. The method has been referred to as extended gradient chromatography[26] and was developed on the principle that if the eluting solution could be slowly and automatically increased from a solution that removes only a few compounds from the ion exchange column to a solution that removes all of the adsorbed compounds from the column, the resulting elution pattern or "profile" could be used to characterize the tissue and as a basis for further separations.

It was shown that in the livers of animals receiving labeled orotic acid the acid-insoluble radioactivity at 12 hours post-injection was almost equal to the acid soluble radioactivity at two hours. [24] Since the radioactivity in the acid insoluble fraction was shown to reside exclusively in uridylic and cytidylic acids it was concluded that "characterization of the radioactive components of the acid soluble fraction of liver should provide valuable information as to the ultimate precursors of the nucleic acid pyrimidines." Hurlbert isolated and characterized 5'-uridylic acid and then the higher phosphates of this compound and by 1954 the entire spectrum of radioactive pyrimidine nucleotides derived from labeled orotic acid were isolated, [25] while in between and among the radioactive pyrimidines the non-radioactive purine nucleotides could be seen.[35] During this time reports of traces of UTP^{30} and GTP^{6} in preparations of ATP and of CTP^{7} in rabbit muscle appeared and it became widely accepted that all of the nucleotides from RNA could be found in the acid soluble fraction of cells in the form of the nucleoside-5'-mono-, di- and triphosphates. The systematic way in which these compounds are eluted from Dowex-1 formate columns is shown in Fig. 2.7.

MONO-	C A G U						COLUMN
DI-		C A	G	U			I
TRI-					C A	G U	
MONO-	C UA G						COLUMN
DI-		C U A G					II
TRI-			C U	A		G	
	0	50	100	150	200	250	FRACTION

Fig. 2.7. Elution of ribonucleoside mono-, di-, and triphosphates from anion exchange columns at pH 2 (column I) or at pH 5 (column II) as a function of the number of phosphate groups and as a function of the base moiety. The letters C, A, G and U represent cytidine, adenosine, guanosine, and uridine, respectively, and the prefixes mono-, di-, and tri-, refer to the number of phosphates on each nucleoside. From Hurlbert, Schmitz, Brumm, and Potter.[26]

In the above studies no clear-cut evidence for either 3' nucleotides or any kind of deoxynucleotides or any new dinucleotides was obtained although many unidentified fractions were present.

Using the same method, R. L. Potter[34] examined thymus tissue and demonstrated the presence of the 5'-mono-, di-, and triphosphates of deoxycytidine and of thymidine but no deoxyribotides of the purines could be found. However, LePage[29] obtained deoxyadenosine-5'-triphosphate from a large quantity of pooled tumor tissue but no evidence for the deoxyguanosine phosphates was reported.

The above reports are strongly supported by the parallel developments in the enzymatic synthesis of the nucleic acids, which in all cases have utilized the 5' nucleotides as substrates. These reports will be described in Chapter VII.

In the few years since the development of the ion
exchange methods for studying the acid soluble
nucleotides, the number of new compounds has
risen steeply, as shown in Fig. 2. 8 which is based
on the review by Henderson and LePage.[23]
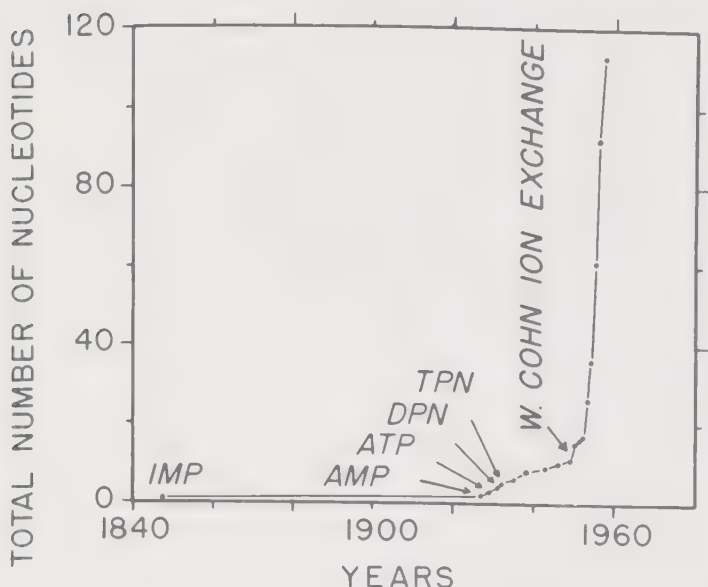
Fig. 2. 8.   The total number of acid soluble nucleotides that
have been discovered since 1840, plotted as a
function of time. Adapted from the review by
Henderson and LePage.[23]

The review describes the isolation and identifica-
tion of over a hundred individual acid soluble nu-
cleotides and notes that during the past four years
new compounds have been added at the rate of about
twenty per year. A recent review on the uridine
coenzymes by Baddiley and Buchanan is[4] a helpful
supplement. Most of the compounds described are
coenzymes and will be discussed in Chapter IX.

The student will also be interested in the chemical syntheses of the nucleotides as reported by Todd in his Nobel Lecture[38] and in the series of chemical syntheses reported by Khorana and his group. [11] Reports of additional nucleotides appearing since the reviews include studies on Drosophila eggs, [39] salmon liver, [40] and growing yeast. [22] In the latter, some 31 previously unknown nucleotides, most of which are peptide derivatives, were noted and correlated with the growth stage of the culture.

H. *Role of the nucleotides in metabolic pathways*

The central role of the 5'-ribonucleotides in nucleic acid metabolism is indicated in Fig. 2.9.
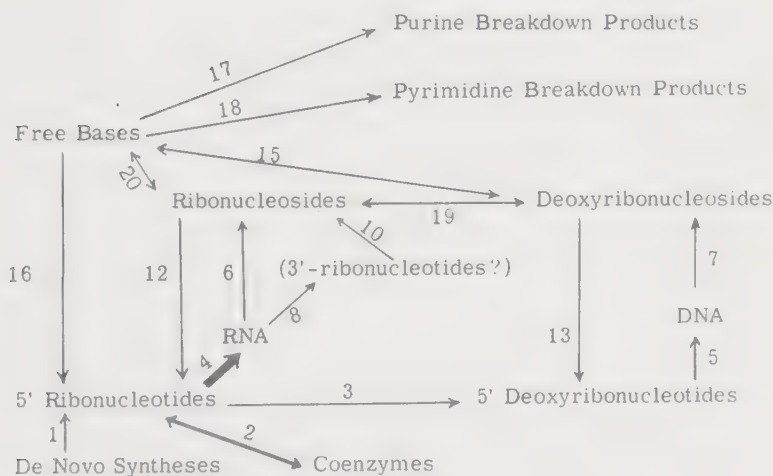
Fig. 2.9. The position of the 5'nucleotides in intermediary metabolism in terms of compounds found in the acid soluble fraction of tissues. The occurrence of the 3' nucleotides in the acid soluble pool has not been demonstrated and they are therefore shown in parentheses. The main directions of flow are indicated by arrows. The "acid soluble fraction" of tissues would include all of the above compounds except RNA and DNA. The numbered pathways will be referred to in greater detail in subsequent chapters.

This figure will also be referred to in Chapters V to IX. It may be seen from the figure that the 5' ribonucleotides represent a metabolic pool which has three separate entrances (pathways 1, 12, and 16 representing de novo synthesis, and synthesis from ribonucleosides and free bases respectively), two main exits (pathways 3 and 4 to deoxyribonucleotides and RNA respectively), and a 6th pathway (to the coenzymes) that is probably closer to an equilibrium than the other pathways. Each of the pathways shown is of course made up of at least four parallel pathways, one for each nucleotide, and in many instances there are numerous branches to the pathways shown.

One of the major unknown features of nucleotide metabolism is whether the 3' nucleotides play any role at all in intermediary metabolism. It is suggested by the chart that they are not involved in any of of the synthetic pathways, according to present knowledge, but we cannot even be sure that they are involved in the breakdown of the nucleic acids outside the digestive tract or possibly in autolysing tissues. The major research involving the 3' ribonucleotides has been the studies on nucleic acid structure, and these have not been concerned with the role of the compounds in metabolism. These studies will be considered in the next chapter (Chapter III) while the metabolic studies will be described in Chapters V-VII. The present chapter has presented the structures and some of the background of the nucleic acid components and we are now ready to take up the structure of the nucleic acids.

REFERENCES

1. ADLER, WEISSMAN and GUTMAN, J. Biol. Chem. 230:717 (1958).
2. AMOS and KORN, Biochem. Biophys. Acta. 29:444 (1958).
3. BADDILEY, in Chargaff and Davidson 1:137 (1954).
4. BADDILEY and BUCHANAN, Quart. Rev. 12:58 (1958).
5. BENDICH, in Chargaff and Davidson, 1:81 (1954).
6. BERGKVIST and DEUTSCH, Acta. Chem. Scand. 7:1307 (1953).
7. BERGKVIST and DEUTSCH, Acta. Chem. Scand. 8:1889 (1954).
8. BROWN and TODD, J. Chem. Soc. p. 44, p. 52 (1952).
9. BROWN and TODD in Chargaff and Davidson 1:409 (1954).
10. CAPUTTO, LELOIR, CARDINI, and PALADINI, J. Biol. Chem. 184:333 (1950).
11. CHAMBERS and KHORANA, J. Am. Chem. Soc. 80:3749 (1958).
12. CHARGAFF, in Chargaff and Davidson, 1:307 (1954).
13. CHRISTMAN, Physiol. Rev. 32:303 (1952).
14. COHEN, Science 123:653 (1956).
15. COHN, Science 109:377 (1949).
16. COHN, J. Am. Chem. Soc. 72:1471, 2811 (1950).
17. COHN, in Chargaff and Davidson 1:211 (1954).
18. COHN, J. Biol. Chem. 235:1488 (1960).
19. COHN and VOLKIN, Nature 167:483 (1951); Arch. Biochem. and Biophys. 35:465 (1952); J. Biol. Chem. 203:319 (1953).
20. DAVIS and ALLEN, J. Biol. Chem. 227:907 (1957).
21. DUNN and SMITH, Nature 175:336 (1955) and Biochem. J. 68:627 (1958).
22. HARRIS, DAVIES and PARSONS, Nature 182:1565 (1958).
23. HENDERSON and LePAGE, Chem. Rev. 58:645 (1958).
24. HURLBERT and POTTER, J. Biol. Chem. 195:257 (1952).
25. HURLBERT and POTTER, J. Biol. Chem. 209:1 (1954).
26. HURLBERT, SCHMITZ, BRUMM and POTTER, J. Biol. Chem. 209:23 (1954).
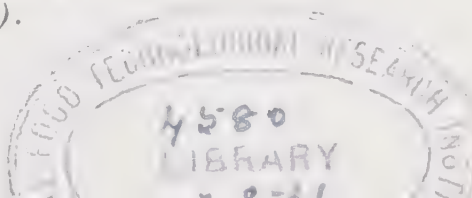
27.   JESAITIS, *Nature* 178:637 (1956).
28.   KEMP and ALLEN, *Biochem. Biophys. Acta.* 28:51 (1958).
29.   LePAGE, *J. Biol. Chem.* 226:135 (1957).
30.   LIPTON, MORELL, FRIEDEN and BOCK, *J. Am. Chem. Soc.* 75:5449 (1953).
31.   LITTLEFIELD and DUNN, *Nature* 181:254 (1958); *Biochem. J.* 68:8 (1958).
32.   MARSHAK, *Proc. Nat. Acad. Sci. U. S.* 37:299 (1951).
33.   PARK, *J. Biol. Chem.* 194:897 (1952).
34.   POTTER, R. L., SCHLESINGER, BUETTNER-JANUSCH and THOMPSON, *J. Biol. Chem.* 226:381 (1957).
35.   SCHMITZ, HURLBERT and POTTER, *J. Biol. Chem.* 209:41 (1954).
36.   SINSHEIMER, *Science* 120:551 (1954).
37.   SINSHEIMER, *Fed. Proc.* 14:282 (1955).
38.   TODD, *Science* 127:787 (1958).
39.   TRAVAGLINA, LEVENBOOK and SCHULTZ, *J. Exp. Cell. Res.* 15:62 (1958).
40.   TSUYUKI, CHANG and IDLER, *Can. J. Biochem. & Physiol.* 36:1185 (1958).
41.   VOLKIN, *J. Am. Chem. Soc.* 76:5892 (1954).
42.   VOLKIN, KHYM, and COHN, *J. Am. Chem. Soc.* 73:1535 (1951).
43.   WYATT, *Nature* 166:237 (1950); *Biochem. J.* 48:581, 584 (1951); *Exp. Cell Research* 3: Supp. 2:201 (1952).
44.   WYATT and COHEN, *Nature* 170:1072 (1952); *Biochem. J.* 55:774 (1953).

Chapter III
Chemical Structure of
RNA and DNA

I. POLYNUCLEOTIDE STRUCTURE AND NATURE OF LINKAGES

II. FACTORS AFFECTING SPECIFICITY OF POLYNU-CLEOTIDE DIESTERASES

    A. End groups on nucleic acids
    B. Specificity in relation to purines and pyrimidines
    C. Preference for C-3'-P or C-5'-P linkages

III. SPECIFIC TYPES OF POLYNUCLEOTIDE DIESTER-ASES

    A. Discussion of alkaline hydrolysis of RNA
    B. Action of Kunitz ribonuclease
    C. Action of snake venom diesterase
    D. Action of other diesterases

IV. SPATIAL CONFIGURATION OF NUCLEIC ACIDS

    A. Brief historical development
    B. Hydrogen bonding in the Watson-Crick model
    C. Construction of paper models
    D. Base pairing in RNA
    E. Hydrogen bonding in RNA double and triple helixes

Chapter III
Chemical Structure of
RNA and DNA

I. *POLYNUCLEOTIDE STRUCTURE AND NATURE OF LINKAGES*

On the basis of present information, the fundamental linear structures of the two types of nucleic acids are the same and consist of long sequences of nucleosides linked together by phosphates in diester linkage from the 3' hydroxyl of one pentose to the 5' hydroxyl of the next pentose, the pentose being D-ribose in the ribonucleic acids and D-2-deoxyribose in the deoxyribonucleic acids[3]. This definition and most previous work leaves unanswered the nature of the respective 3' and 5' hydroxyls on the two ends of the nucleic molecule, but in the examples to be illustrated, we shall indicate a phosphate monoester on the 5' end and a free hydroxyl group at the 3' end as in the case of the product of ribopolynucleotide phosphorylase[20]. It has already been indicated that the classical RNA contains the four bases adenine, guanine, cytosine and uracil, while the classical DNA contains adenine, guanine, cytosine and thymine. It was also indicated that alternative purines and pyrimidines have recently been discovered.

Since to draw the structures or to think about certain aspects of nucleic acid chemistry is unnecessarily cumbersome if the entire structure is employed, it will be advantageous for the student to learn and use the conventional "shorthand" structures employed by Markham and Smith, Ochoa, Heppel, Singer and others in the form in which it has recently been accepted by the

Editors of the Journal of Biological Chemistry. An intermediate condensed form will also be shown[3]. In all cases the structures are aligned in the fashion already shown for the free nucleosides (Fig. 2.5) and nucleotides (Fig. 2.6) thus the "5' end" will be at the left of the reader and the "3' end" will be at the right of the reader. In Fig. 3.1 is shown an oligonucleotide with the structure of a ribo-polynucleotide together with two shorthand forms of the same molecule. The conventional structural representation includes the indications for the 3-dimensional structure in that the $5'-CH_2$ on C-4' and the N on C-1' are oriented up and the C-2' and C-3' hydroxyls are oriented down from the horizontal D-ribofuranose ring. For the present we will not be concerned with the 3-D structure of the nucleic acids but will consider the structure as deduced from the action of hydrolytic enzymes and of dilute alkali, omitting a discussion of the classical studies by Khorana[20] in which chemical syntheses of oligonucleotides have been carried out.

The structure of ribonucleic acid is believed to be identical with that shown in Fig. 3.1 except for the number of repeating units, which is one to two orders of magnitude greater than in the structure shown.

The single-strand structure of deoxyribonucleic acid is also believed to be identical with that shown in Fig. 3.1, except for the number of repeating units, plus the additional fact that the 2'OH is replaced by H, thus eliminating the possibility of forming a cyclic 2', 3'-phosphate. Thus the polynucleotide structure of DNA is based on the same 3', 5'-phosphate diester linkage that is believed to form the basis of RNA structure. The 3-D structure of DNA will be considered later.

The accepted shorthand version for describing polynucleotide structures is most useful for discussing the action of hydrolytic enzymes as well as the nature of their specificity. It can be used for mononucleotides
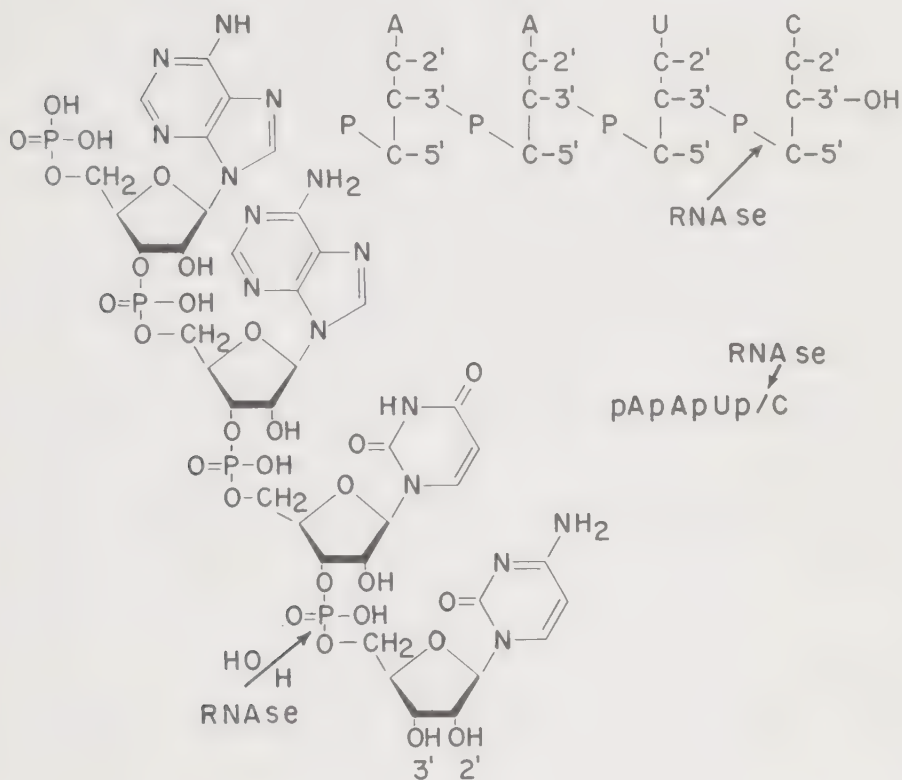
Fig. 3.1.   The detailed structure of a short oligoribonucleotide with typical polyri-
bonucleotide linkages. The example contains adenosine, adenosine,
uridine, and cytidine, with guanosine deliberately omitted to avoid any
implication of a repeating tetranucleotide structure. There are three
3'-5'-phosphate diester linkages, a 5'phosphate monoester on the "5' end",
and a 3'-OH on the "3' end" of the structure. The same structure has also
been represented by two "shorthand" structures in current use. By compari-
sons of the structures the student can readily see what has been omitted in
the abbreviated structures and what has been implied in them. In many
cases the letters B (for Base = either purine or pyrimidine), PU (for
purine), and PY (pyrimidine) can be used instead of letters representing
individual purines or pyrimidines. In the case of the deoxyribonucleotides
the short form is prefixed by the letter d.

as well as the longest polynucleotides that can be de-
scribed in terms of sequence. It follows from Fig. 3.1
that according to convention pA would be the abbrevia-
tion of adenosine 5'-monophosphate (5' AMP) and Ap
would be the abbreviation for adenosine 3'-monophos-
phate (3' AMP).

II. FACTORS AFFECTING SPECIFICITY OF POLYNUCLEOTIDE DIESTERASES

The enzymes that hydrolyze the nucleic acids to mono-
nucleotides have only recently begun to be purified and
the number of reagent-pure enzymes is still very
small. However, these enzymes have more than in-
trinsic interest since they can be used to unravel many
of the features of nucleic acid structure[3], especially
since the advent of chemically synthesized oligonucleo-
tides[20]. Their specificities involve the following cate-
gories:
> (1) Presence of phosphate or hydroxyl end groups.
> (2) Presence of purines or pyrimidines around the
>   phosphate diester.
> (3) Preference for attacking the C-3'-P or the
>   C-5'-P linkage.

A diesterase will attack only one of the two diesters
and will not attack monoesters. In describing the action
of the diesterases, the short form of the polynucleotide
formulae as shown in Fig. 3.1 is preferable and the ac-
tion can be easily described by a bar in the proper
place, as in the example given.

A. End groups on nucleic acids

If we attempt to list the variations in nucleic acid
structure that are theoretically possible in terms
of end groups the number is at least five, and
probably greater. These possibilities will be list-
ed below, using the simplest possible dinucleotide
as a model, i.e. BpB (cf. Fig. 3.1). It seems
necessary to advocate a new term for such a nucleo-

tide and it is here proposed that whenever both ends of an oligo- or polynucleotide are the same with respect to phosphate or hydroxyl the prefix ambi- should be used. The various possibilities are thus:

Descriptive Name Indicating End-Groups	Simplest Example	
Ambi-OH (oligonucleotide):	BpB	
Ambi-phosphate	pBpBp	(corresponding mononucleotide would be 3', 5' diphosphate).
5' phosphate:	pBpB	(implies 3' is OH)
3' phosphate:	BpBp	(implies 5' is OH)
2', 3' cyclic phosphate:	BpB -	cyclic p (cf. J. Biol. Chem. in which the form BpBp'. introduced by Markham and Smith is not employed)

All the above forms have been obtained by various means and have been used as substrates to test the nature of the enzymatic specificities. Theoretically possible are several additional forms which have not as yet been described. Thus one might expect that the 5' phosphates (example: pBpB) might be converted to 5' diphosphates or triphosphates by the action of ATP or some other donor:

5' diphosphate:	ppBpB
5' triphosphate:	pppBpB

It is already known that certain types of RNA (soluble RNA=S-RNA) are able to transport amino acids, apparently on the 3'-OH end:

3' Amino Acyl: BpB–Amino Acyl (length and
                                        specificity
                                        not fully
                                        known, see
                                        Chapter XI)

The presence of a 3'-phosphate would presumably block the acceptance of the amino acid.

The above discussion provides some basis for believing that the end groups of the nucleic acids may represent important sites for determining enzyme action and for the regulation of nucleic acid function. For example, the alkaline phosphatase in tissues has long been studied without knowing what its true substrate may be. The action of this enzyme on the terminal phosphates of specific polynucleotides might be of interest.

B.   *Specificity in relation to purines and pyrimidines*

The action of the ribonucleases varies considerably with respect to the presence of purines or pyrimidines along the chain. The Kunitz ribonuclease will attack a diester between two pyrimidines, or a PYpPU diester but it will not attack a PUpPU or a PUpPY diester. The snake venom diesterase attacks all diester linkages regardless of the purine or pyrimidine attachment.

C.   *Preference for C-3'-P or C-5'-P linkages*

In addition to the question of what base is attached to the sugar, there is an intrinsic property of the various diesterases to attack one or the other esterified group in a phosphate diester, and to leave

the remaining monoester intact. Thus Kunitz ribonuclease attacks the C-5'-P linkages exclusively and yields products with 3' phosphate ends, while the snake venom diesterase has the opposite action and attacks all C-3'-P linkages, yielding products with 5' phosphate ends.

With the above discussions of the variables in diesterase action we may now consider individual examples that combine all of the listed parameters.

III.   SPECIFIC TYPES OF POLYNUCLEOTIDE DIESTERASE ACTION[3], [16]

A.   Discussion of alkaline hydrolysis of RNA

Much attention has been given to the action of dilute alkali (0.1 to 1.0 N) on nucleic acids at temperatures between room temperature and 80°C and for periods of from 20' to 24 hours at the lower temperatures. RNA is completely degraded to a mixture of 2' and 3' nucleoside monophosphates, while DNA is not attacked but is merely solubilized[3]. In RNA the initial action is to convert each diester to a cyclic 2', 3' ester, which is then hydrolyzed to a mixture of the 2' and 3' monoesters, written 2'(3'), while any 3' monophosphates (Bp) coming from the 3' terminal nucleotide will not be converted to 2' esters[3]. If the ribo-polynucleotide had a terminal 3'-OH this group will appear as a nucleoside. Similarly, if there was a terminal 5'-phosphate, this group should appear as a mixture of 2', 5'- and 3', 5'-diphosphates.

Examples of the action of alkali on polynucleotides are as follows, with bars inserted in the shorthand formulae to show the points of attack:

1.   BpBpBpB becomes Bp/Bp/Bp/B to yield initially 3 B-cyclic p + B, and finally 3 B-3' (2')-monophosphates + B.

2. BpBpBpBp becomes Bp/Bp/Bp/Bp to yield initially 3 B-cyclic p + Bp, and finally 3 B-3' (2')-monophosphates + Bp.

3. pApApUpC (see Fig. 3.1) becomes pAp/Ap/Up/C to yield initially pA-cyclic p + A-cyclic p + U-cyclic p + C, and finally, adenosine-2' (3'), 5'-diphosphate + adenosine-2' (3')-monophosphate + uridine-2' (3')-monophosphate + cytidine.

Alkaline hydrolysis has been used to further characterize the products of enzyme action on RNA, since any diesterase linkages not attacked by the enzyme will be split by alkali, as will be shown below.

B. *Action of Kunitz ribonuclease*[3, 16]

The heat-stable crystalline ribonuclease isolated from pancreas by Kunitz is the best known of the diesterases and may be characterized as follows:

1. Nonspecific as to presence of end groups.
2. Splits only between C-5' and P of diesters in which the C-3' is attached to a pyrimidine, i.e. PYp/PU or PYp/PY but not PUpPY or PUpPU.
3. For the linkages attacked, the action is similar to that of alkali in that cyclic 2', 3'-monophosphates are transiently produced. This reaction mechanism may account for the absence of any action by alkali or Kunitz ribonuclease on DNA, since in the absence of 2'-OH groups, the cyclic intermediates cannot be formed. The action differs from that of alkali in that alkali hydrolyzes the cyclic phosphates randomly to a mixture of 2'- and 3'-monophosphates, while ribonuclease hydrolyzes to 3' monophosphates specifically. Dilute acid converts either 2' or 3' monophosphates to an equilibrium mixture, but alkali does not change the monophosphates once produced[3].

4. On the basis of the above properties, the final product of ribonuclease action will consist of the following, taking an example in which the initial RNA was ambi-OH ended and the 3' end was a purine nucleoside adjacent to a pyrimidine (thus, RNApPYpPU):

    a.   PU (nucleoside, from the 3' end).
    b.   PYp (one for every PUpPYpPYp or PYpPYpPUp sequence)
    c.   PUpPYp
    d.   PUpPUpPYp
    e.   PUpPUpPUpPYp
    f.   PUpPUpPUpPUpPYp etc.

    Thus every unbroken sequence of <u>purine</u> residues will be found intact when ribonuclease action is complete, and each one will be terminated by a PYp on the 3' end. Further evidence supporting this explanation of ribonuclease action has come from studies in which ribo-poly-nucleotide phosphorylase (Chapter VII) was used to synthesize long sequences of pyrimidines on short "primers" consisting of purine ribonucleotides. When a polymer such as pApApUpUpU....pU was treated with ribonuclease, the pyrimidines were digested off until the pyrimidine next to the purine primer was reached, yielding pApApUp[16, 30].

5. A typical example of ribonuclease action is shown in Fig. 3.1 in which pApApUpC is indicated to be vulnerable at only one site, namely the Up/C linkage. The pApApUp residue is analogous to fragment d in the preceding paragraph and differs only in the possession of a 5' phosphate end group.

6.   The products of ribonuclease action may be subjected to further treatment with other agents. Thus the oligonucleotides c to f in paragraph 4, if treated with a phosphate monoesterase, such as the prostatic phosphatase, will yield 3' OH ended products, and these treated in turn with alkali would yield a series of purine-3' (2')-monophosphates and a pyrimidine nucleoside. Or if snake venom diesterase were used without mono-esterase pretreatment, the products from each fragment would be a purine nucleoside, some purine nucleoside 5'-monophosphates, and a 3',5'-pyrimidine nucleoside diphosphate. The action of snake venom diesterase will be discussed further in the next section.

C.   *Action of snake venom diesterase*[3, 16, 26]

The venom of various snakes including the rattlesnake <u>Crotalus</u> <u>adamanteus</u> and Russel's viper has become a valuable reagent in nucleic acid chemistry, and so far has certain properties not shared with any other enzyme. It is to be hoped that a more abundant source of a similar enzyme will be discovered, and made available in a highly purified form. The snake venom diesterase is also a ribonuclease and unfortunately has been referred to as such, thus confusing it with Kunitz ribonuclease. It acts on RNA without previous treatment and on DNA that has been reduced to oligonucleotides by previous treatment with DNAse I. Crude lyophilized snake venom contains a 5'-phosphate monoesterase in addition to the 3', 5' diesterase. If the monoesterase is not removed, it will dephosphorylate the 5' esters to nucleosides as soon as they are formed by the diesterase. If dialyzed crude snake venom is purified by the method of Hurst and Butler[17], the 5' monoesterase can be separated from the diesterase to give a very useful preparation. Further purification has been accomplished by Razzell and Khorana[26]. The results to be presented

reveal certain abilities at the lowest concentration, with additional properties when the amount of enzyme is increased 20x or 1000x[16]. These apparently are not due to impurities[26]. These properties are particularly relevant to the question of endgroup specificity. The characteristics of the snake venom diesterase are as follows:

1. Non-specific as to purines or pyrimidines.
2. Always splits between C-3' and P to give C-5' phosphates.
3. Acts preferentially on oligonucleotides with 5'-end phosphate and 3'-OH, but splits diesters sequentially beginning at the 3' end, yielding mononucleotides.
4. Substrates with 3'-end phosphate acted on slowly or not at all except with 1000x enzyme concentration, and are strong competitive inhibitors. As substrates, the first products are oligonucleotides (see 5c, below).
5. Examples of action:
   a. On 5'P ribo-polynucleotides: pB/pB/pB yields pB+pB+pB.
   b. On Ambi-OH ribo-polynucleotides: B/pB/pB yields B+pB+pB (20 fold increase in enzyme concentration).
   c. On 3'P ribo-polynucleotides: BpBpB/pBpBpBp split at linkage away from the 3' end to yield Ambi-OH (see b) and Ambi-P (see below) with 1000-fold enzyme.
   d. On Ambi-P ribo-polynucleotides: pBpB/pBpBpBp split at linkage away from 3' end (with 1000-fold enzyme) to yield 5'P-ended preferred substrates which quickly convert to 5'-monophosphates as in a and slowly convert progressively smaller ambi-P-ended fragments to 5'P-ended nucleotides and one 3',5'-diphosphate for every ambi-P-ended diester.

e.   Note that every RNA molecule bearing a
      3'P should yield one 3'5'-diphosphate when
      large amounts of enzyme are used.   How-
      ever, if any ribonuclease of the Kunitz
      type is present even in very small amounts
      in the RNA preparation or in the snake
      venom, the tendency will be to produce
      fragments of the type shown in c to f in
      section 4, a series of purine nucleoside
      diesters terminating in a pyrimidine 3'
      phosphate and each of the latter will yield
      a pyrimidine 3', 5'-diphosphate according
      to the above scheme[8], while some termi-
      nal pyrimidine 3'-phosphates and nucleo-
      sides would be liberated as in a and b of
      section 4.   These products were all found
      in a snake venom diesterase digest of RNA
      by Cohn and Volkin[7] who discussed the data
      in terms of branching of RNA polynucleo-
      tides. The present opinion in the field is
      that no unequivocal evidence for branched
      polynucleotides is available since there
      may be other explanations for the occur-
      rence of pCp and pUp in RNA digests.
6.   The above characteristics seem to apply with
      equal force to polyribonucleotides and DNA
      oligonucleotides[16, 26].

D.   *Action of other diesterases*[16]

1.   Enzymes attacking C-3'-P to yield 5' phos-
      phates. In addition to the snake venom dies-
      terase, this group includes pancreatic DNAse
      I which acts on DNA molecules too large for
      the snake venom diesterase and gives fragments
      acted upon by the latter enzyme. The pancre-
      atic DNAse I will not act on dinucleotides (ex-
      amples used were deoxy-ApCp, pCp, TpGp and
      ApC) but was able to hydrolyse the trinucleo-
      tide deoxy-ApApTp which was converted to
      deoxy-ApA and deoxy-pTp, suggesting prefer-
      ential attack on the PUpPY sequence[25].

2. Enzymes attacking C-5' to P to yield 3' phosphates. Several enzymes showing this type of action include splenic DNAse II, thymic DNAse II and a micrococcal DNAse. A heat stable RNAse has been isolated from spleen by Kaplan and Heppel[19] and appears to be very similar to Kunitz ribonuclease, in contrast to the spleen diesterase isolated by Heppel and Hilmoe[15]. The latter enzyme acts on both RNA and on deoxyribo-oligonucleotides in a manner quite the opposite of the snake venom diesterase[16]. The products are 3' monoesters and the 3' P-ended diesters are preferred substrates while the 5' P-ended diesters are resistant, but it resembles the venom diesterase in being nonspecific as to the bases. Ribonucleases from tobacco leaf[27] and from rye grass[29] have been described as differing from Kunitz ribonuclease in attacking all diester linkages to give cyclic 2', 3' phosphates of both purines and pyrimidines. The rye grass enzyme[29] hydrolyzes both purine and pyrimidine cyclic phosphates to the 3' monophosphates and is not affected by the nature of the end groups. The tobacco leaf enzyme[27] hydrolyses only the purine cyclic phosphates. The enzymes mentioned have been used to study the structure of nucleic acids[29] and will no doubt become increasingly useful as their purity and specificities progress.

IV. SPATIAL CONFIGURATION OF NUCLEIC ACIDS

The structure of the nucleic acids can only begin to be appreciated from a formulation such as shown in Fig. 3.1, which only vaguely suggests a helix even to those who are aware of the Watson-Crick theory and who have seen a three-dimensional (3-D) model. The Watson-Crick Theory was not an obvious one and it did not arise in a single step. Although structural considerations

could suggest a helix from information given in Fig.
3.1, the previous ways of depicting nucleic acid struc-
tures and the many bonds permitting free rotation did
not immediately lead to the presently accepted 3-D con-
figuration.

If the projections around the β-D-ribofuranose in the
RNA of Figure 3.1 can be visualized, the first approxi-
mation of the DNA structure will be seen. If lines are
drawn through each pentose from 1' to 4' and from 2' to
3' and the paper is folded down on the 2'3' axis and up
on the 1'4' axis according to convention, one can then
place the figure or a copy of it in such a way that the
planes of the purine and pyrimidine bases are in a hori-
zontal plane, making cuts where needed. The resulting
configuration will begin to approach the Watson-Crick
helix, which, given a vertical axis, will show the planes
of the bases as horizontal and the planes of the pentoses
nearly vertical but in addition the Watson-Crick model
will show a second helix spiralling with a fixed relation
to the first, as in Fig. 3.2. The concept of the double
helix based on specific purine-pyrimidine interaction
was the real element of novelty in the 3-D structure
and was not apparent from considerations such as those
mentioned in connection with Fig. 3.1. It is of interest
to review some of the steps leading up to the type of
structure shown by the model in Fig. 3.2.
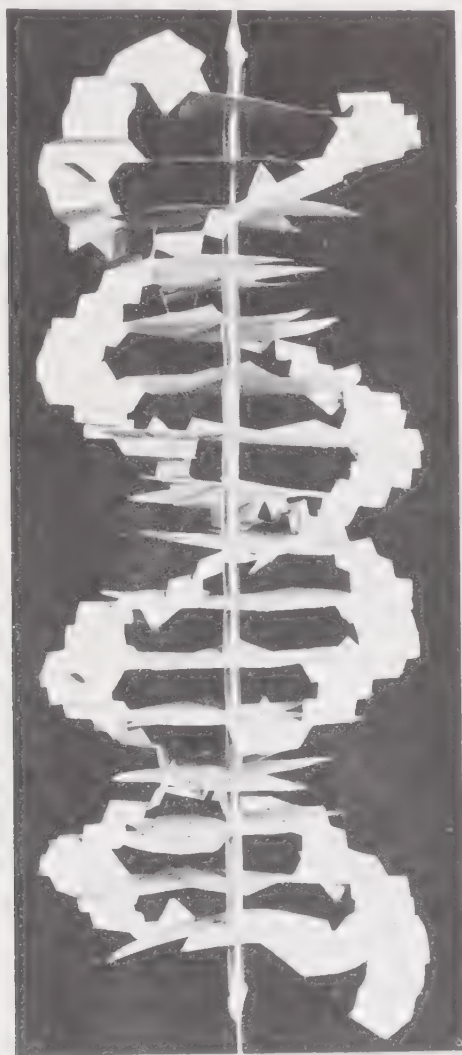
Fig. 3.2. A three-dimensional model of the double-helix structure of DNA,
designed and constructed in accordance with the theory of Watson and
Crick[35]. This model can be constructed from cardboard representations
of paired nucleotides using the templates provided in the four figures that
follow this one, or it can be constructed from 16 such nucleotide pairs
that are available in color-coded die-cut form in a kit (DNA Model Kit,
Van R. Potter, Burgess Publishing Company, Minneapolis, 1959,
price $1.00). The model shown in the photograph has a diameter of six
inches, and the planes of the nucleotides are one inch apart, with a scale
of 3.4 Ångstrom units per inch. All such models fail to suggest the enor-
mous length of the DNA molecule. This dimension can be introduced by
pointing out that a true model of a small DNA molecule with 6000 pairs of
nucleotides would be 500 feet high if represented by the units used in the
above model.

A.   Brief historical development

1.   In 1938 Astbury and Bell[1] reported that X-ray diffraction measurements on DNA fibers showed a regular spacing of about 3. 34 Å along the fiber axis, which was interpreted as a succession of flat nucleotides standing perpendicular to the axis. It was assumed at that time that the sugars and the bases were in the same plane.

2.   During 1949-1952 Furberg[12] proposed that the planes of the sugar rings were parallel to the long axis of the DNA molecule while the bases were at right angles to the sugars and to the long axis, essentially as in the current manner, with the long axis oriented into a spiral.

3.   In 1953 Pauling and Corey[24] proposed a helical structure which consisted of three intertwined polynucleotide chains but this formulation placed the phosphates on the inside and the bases on the outside, which according to Jordan[18] neglected titration data by Gulland, Jordan and Taylor[14] which had provided evidence ᵗhat the phosphate groups were accessible while the -NH-CO- groups on the bases did not titrate in the expected manner, and behaved as if participating in hydrogen bond interaction.

4.   During 1950-1953 Chargaff and coworkers[4, 5] accumulated data showing marked regularities in the ratios of total purines to total pyrimidines, of adenine to thymine, of guanine to cytosine, and of adenine plus cytosine to thymine plus guanine groups.

5.   In 1953 Watson and Crick[35] combined all of the available data in a double helix theory in which the two helical chains are each coiled around the same axis with their components arranged linearly but in opposite directions. The pyrimidines and purines were put on the inside of the helix and the phosphates on the outside.

A pair of nucleotides occurs in the direction of the long axis every 3.4Å. The structure repeats every 10 nucleotides or 34Å, the angle between adjacent nucleotides in the same chain being assumed to be 36°.

The most attractive feature of the postulated structure is the hydrogen bonding which is in complete agreement with the analytical data of Chargaff et al mentioned above. It is assumed that for every thymine in one chain there is an adenine hydrogen-bonded to it in the adjacent chain and for every guanine in one chain there is a cytosine (or cytosine derivative) in the opposite chain. Thus each purine is opposite a pyrimidine in every pairing and from studies with models it appears that two pyrimidines will not bridge the gap while two purines would be unable to enter the available space. The significance of the adenine-thymine and guanine-cytosine pairing will be discussed further in Chapter X.

6.   In 1955 Grunberg-Manago and Ochoa[13] discovered polynucleotide phosphorylase, an enzyme that would synthesize quantities of polyribonucleotides from a single type of nucleoside diphosphate or from mixtures of nucleoside diphosphates. The former are referred to as poly A, poly U, poly C, and poly I. In all cases the products have the general formula $(pB)_x$, i.e., 5' phosphate ended, and are considered models of RNA internucleotide structure. The great value of these synthetic RNA molecules containing only one base is the new impetus they have given the theoretical and experimental approaches to the problem of the 3-D structure of the nucleic acids (see below).

7.   In 1956 Warner[34] showed that the ultraviolet absorption of a mixture of poly A and poly U is considerably less than that of the individual polymers or of the component nucleotides.

This is referred to as the hypochromic effect. In addition the molecular weight and viscosity increased on mixing and it was suggested that pairing of poly A with poly U molecules was occurring.

8. In 1957 Rich and Davies[28], also using the Ochoa polymers, extended Warner's results by showing that the X-ray diffraction pattern of fibers made from poly A and poly U complexes was similar to that of DNA. Similar complexes were found between poly I and poly C. Felsenfeld, Davies and Rich[11] showed evidence that triple stranded structures could be formed from poly A and poly U in the presence of magnesium ions. The authors noted that the triple helix might be a model for a combined DNA double helix with a matched RNA single strand.

9. In 1958 Stent[32] and Zubay[36] continued the theoretical discussion of the triple helix concept and at present it seems likely that we may eventually see some kind of a triple helix containing both RNA and DNA and retaining the base-pairing of the Watson-Crick DNA double helix with some new rules for transferring this information to an RNA molecule.

10. In 1959 the existence of DNA in single strands in certain types of bacteriophage such as Phi X-174 became established by Sinsheimer[31], and by Tessman[33] and this information will undoubtedly have to be integrated with the double helix and triple helix concepts.

It is now evident that the student will need to thoroughly understand the hydrogen bonding between base pairs in the double helix in order to have a foundation for future developments in triple helix and single strand structure and function.

B.   Hydrogen bonding in the Watson-Crick model

The analytical data from Chargaff's group provided evidence that in a wide variety of DNA preparations, which were conceded to be mixtures of many individual DNA molecules, the ratio of adenine to thymine and the ratio of guanine to cytosine was so close to 1.00[4] that they must be reckoned with in any theoretical consideration of DNA structure, and any analytical deviations from the figure now have to be suspected of containing single strands of DNA without the complementary single strands. Indeed, it is of great theoretical interest to find that the DNA from the bacteriophage Phi X, which is single stranded by several criteria, turns out to have A/T and G/C ratios of 0.75 and 1.3 respectively deviating markedly from the usually observed ratios of 1.0 for each[31].   The only suggestion that permits an understanding of the analytical regularity in previous mixtures of DNA molecules is the concept that each strand is matched by a complementary strand in which the complementarity rigidly adheres to the A/T and G/C pairings, with the result that no matter how complex the mixture, the analytical data maintain the ratios.   The finding that single strand DNA does not have the ratios found in most DNA samples is further support for this concept.

In seeking an explanation for the rigid stoichiometry of one thymine per adenine and one guanosine per cytosine, Watson and Crick[35] proposed the hydrogen bonding that is illustrated in Figs. 3.3-3.6.

Fig. 3.3. The cytosine-guanine (CG) pair of deoxyribonucleotides showing hydrogen
bonding (dotted lines). The figure is also designed to serve as a template
for constructing CG pairs for a 3-dimensional model as shown in Fig. 3.2.
The template is designed to give the correct β-D-deoxyriboside configura-
tions if properly folded as shown. The dotted lines around the oxygen of
the furanose ring should be slit to permit the pentose to lie at right angles
to the plane of the purine and pyrimidine bases when the model is folded
on the dashed lines. The model should also be cut along the curved lines
between the 36° markers and the phosphates before folding. If each
successive pair of nucleotides is turned through 36° and mounted at a one
inch interval above the previous pair on a vertical wire as in Fig. 3.2,
the 5'-phosphates of the successive pairs can be cemented over the 3'OH
of the deoxyribose of the adjacent nucleotide to complete the 3',5'-phos-
phate diester linkage. (Effective spacers can be made from soda straws,
cut in one inch lengths.)
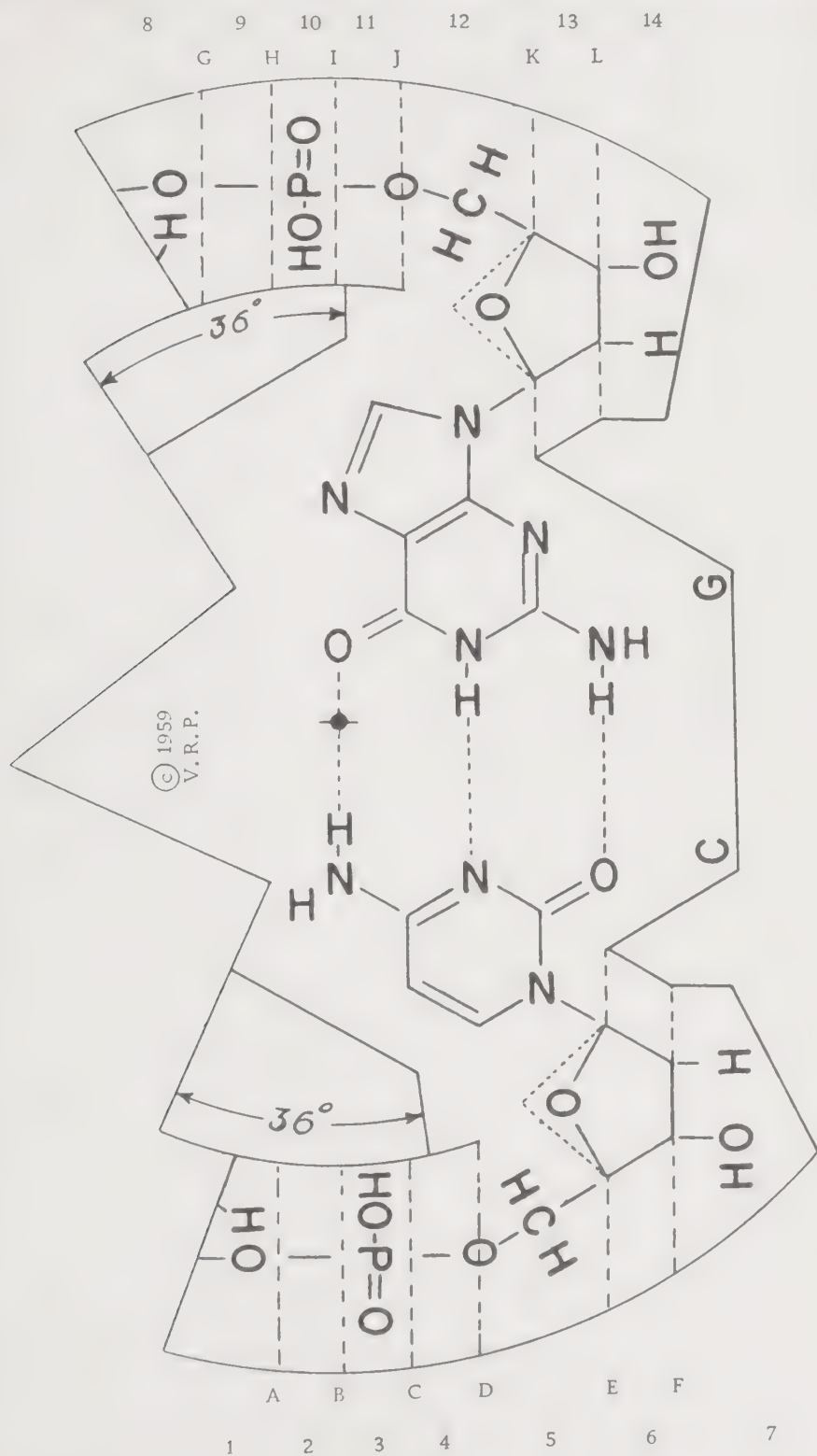
Fig. 3.4. The guanine-cytosine (GC) pair of deoxyribonucleotides showing hydrogen
          bonding. For use as a template for 3D models see instructions for Fig. 3.3.
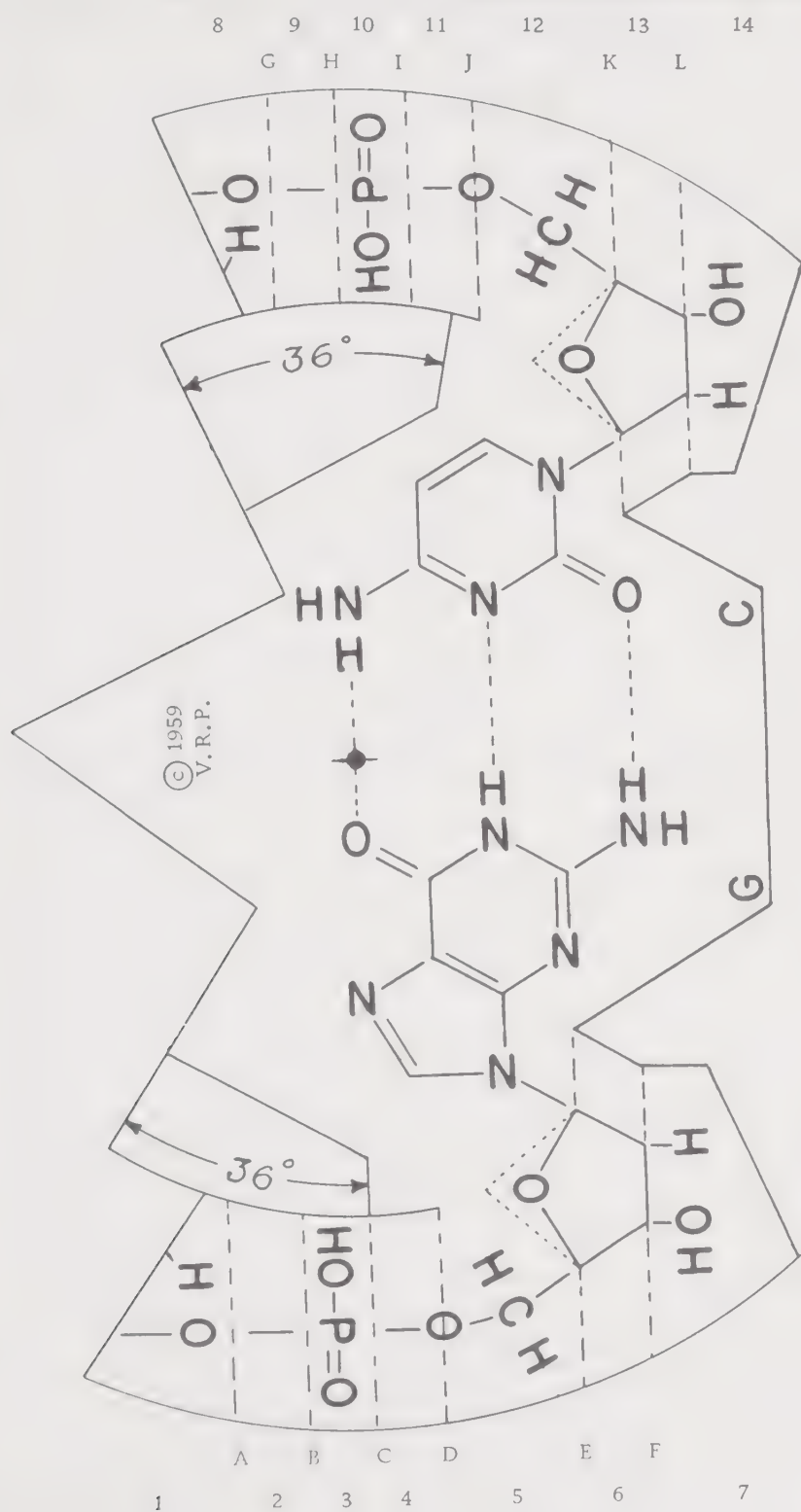
Fig. 3. 5.   The thymine-adenine (TA) pair of deoxyribonucleotides showing hydrogen
bonding.   For use as a template see Fig. 3. 3.
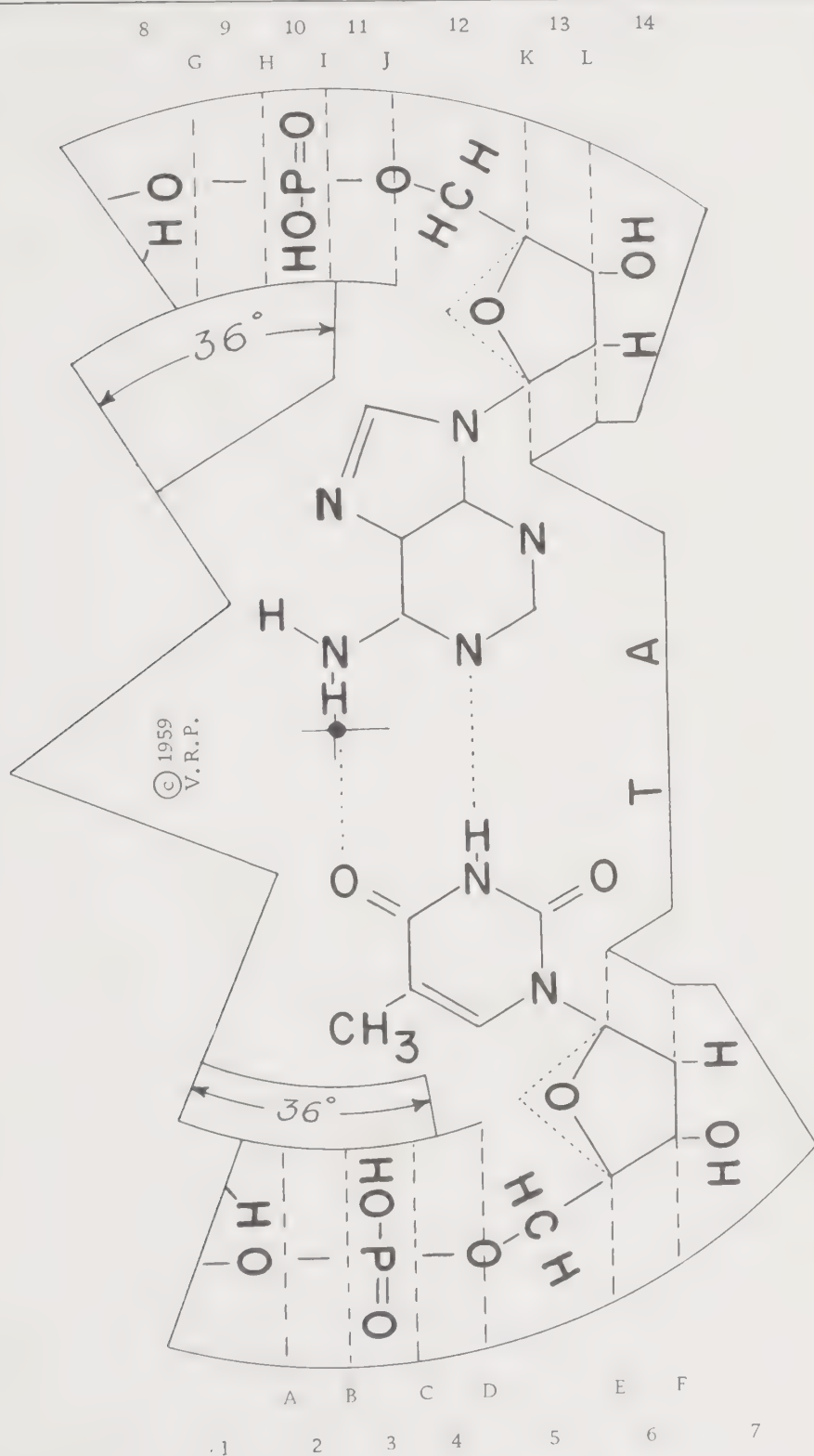
Fig. 3.6. The adenine-thymine (AT) pair of deoxyribonucleotides showing hydrogen
       bonding. For use as a template see Fig. 3.3.
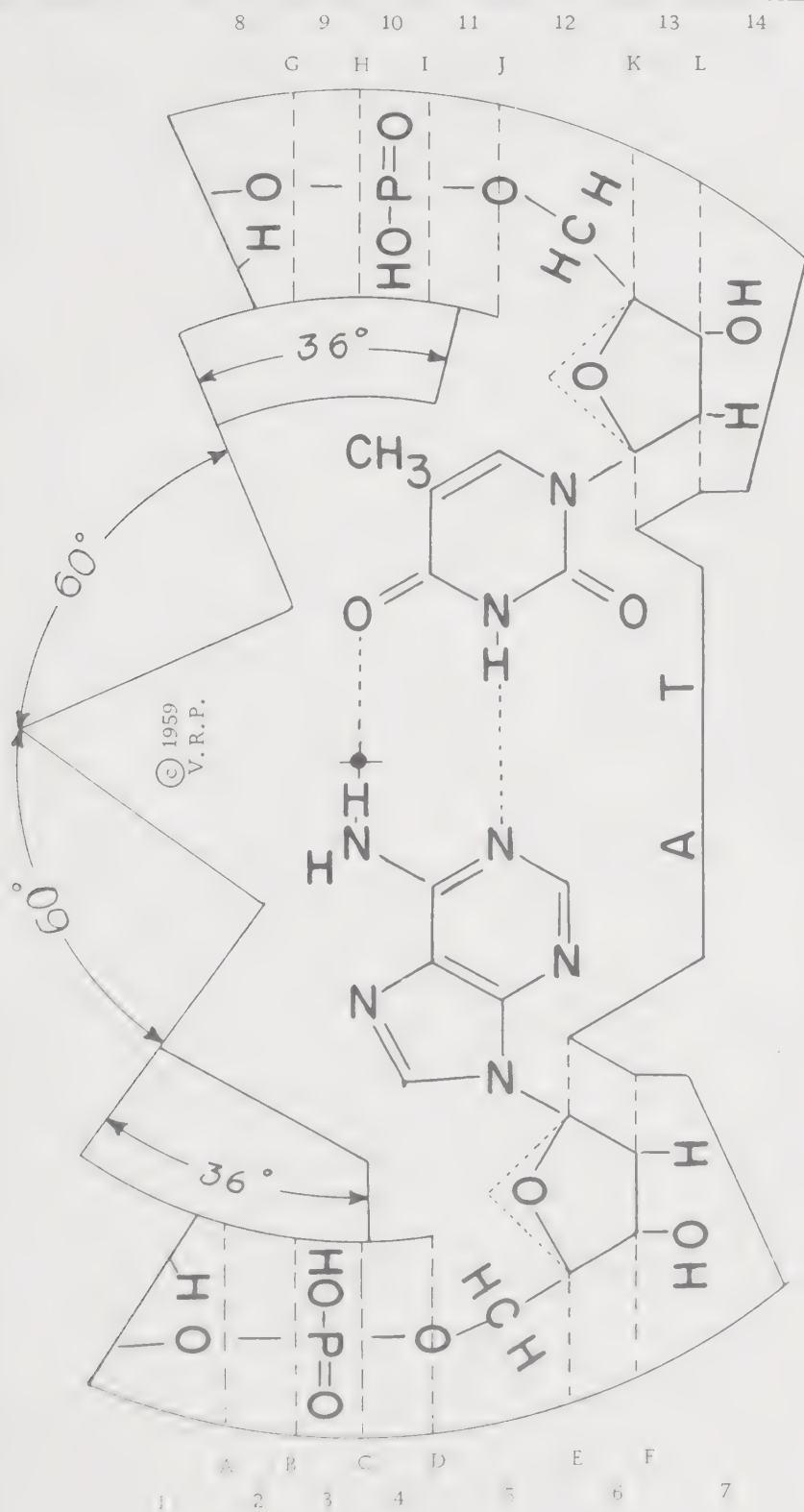
There are only two purine: pyrimidine pairs that meet all the geometric requirements of a DNA double helix, namely adenine opposite thymine, and guanine opposite cytosine. However, each of the four bases can occur in either chain, and the geometric requirements are such that all the bases in the paired chain are oriented in the opposite direction. The four possible pairings in DNA can be listed with the numbers of the ring carbons or nitrogens that are closest to the hydrogen bonds written opposite the corresponding number in the paired base:

	4	*	6	
	3		1	
cytosine	2		2 guanine	(Fig. 3.3)
	6	*	4	
	1		3	
guanine	2		2 cytosine	(Fig. 3.4)
	4	*	6	
thymine	3		1 adenine	(Fig. 3.5)
	6	*	4	
adenine	1		3 thymine	(Fig. 3.6)
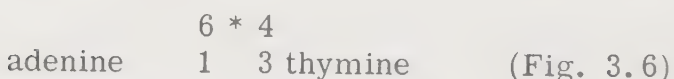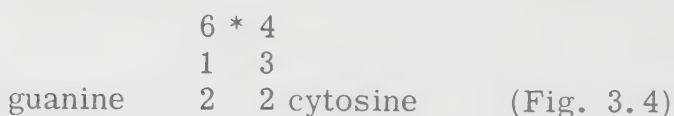
It should be noted that the Chemical Abstracts numbering system gives numbers 3 and 4 to the pyrimidine ring atoms numbered 1 and 6 respectively in the older system. In the above pairings, the asterisk shows the approximate location of the long axis of the helix. In the cytosine-guanine pairings, the figures show a third bonding between cytosine and guanine, as suggested by Pauling and Corey[23]. The spatial relationships can be understood much better if the structures shown in Figs. 3.3-3.6 are used to construct a 3-D model of a Watson-Crick double helix as shown in Fig. 3.2. These figures describe the spatial relationships for the four pos-

sible base pairings found in the double helix, and can be used as templates for model construction.

C.   *Construction of Paper Models*

The layout of the templates shown in Figs. 3.3-3.6 will now be described, and in so doing, the approximate structure of the paired nucleotides and of the double helix will be brought out.

The construction of paper models of the double helix is feasible in the first place because the bases are planar and in this structure lie in approximately the same plane as the C-1' and C-4' carbons of the pentoses to which they are directly attached, while the planes of the pentoses are at nearly right angles to the plane of the bases. The model presents the pentose ring as planar although it is actually understood to deviate somewhat from planarity.

The templates were laid out on a special type of paper ruled in hexagonal pattern with point to point contact between the hexagons, so that each hexagon is bounded by six equilateral triangles. The pyrimidine ring in each figure coincides with a hexagon, and was drawn first. In Fig. 3.3 this was cytosine. The purine guanine was then drawn in the hydrogen-bonded position, as shown. In each figure, the base at the bottom of the page (or left-hand base as one faces the center of the helix) is reversed relative to the opposing base in order to permit hydrogen-bonding with the opposite base. (Compare with Figures in Chapter II).

Assuming that the fundamental structure is a triple helix, an equilateral triangle was drawn taking as the base of the triangle the line between the centers of the glucosidic nitrogens in the pyrimidine and the purine of the double helix. The apex of the triangle is the only part shown in the figure and is to be

used to position a single purine or pyrimidine nucleotide in modeling the Stent or Zubay RNA member of the triple helix.

The long axis of the double helix was then taken to be the center of the triangle with its apex occupying a 120° sector of the circular cross-section of the double helix, i.e., the "deep groove" of the double helix, to be occupied later by the third member of the triple helix.

The deoxypentose molecules were then drawn in nucleoside linkage. Since one member of the base pair had to be drawn in the reversed form as compared to the usual form, in order for the hydrogen bonds to match, the pentose molecules have to be carefully oriented in order to keep the bases in β configuration. This is provided for by drawing a line between the C-1' and C-4' of each pentose and folding the paper up from the plane of the lower base (cytosine in Fig. 3.3) and down from the plane of the upper base on each page (guanine in Fig. 3.3). The paper should be folded to place the pentose planes at right angles to the base planes. The 3' OH group on each pentose would then be folded outward to give the correct D-configuration in each case.

The phosphates were drawn attached in ester linkage to each 5' OH and should be folded so that the lower one is slightly below the plane of the bases and the upper one is slightly above this level.

We can now discuss the assembly of the polynucleotide structure. With the four templates here available, it is possible to make a polynucleotide model with as many mononucleotide units as may be desired. Sixteen base pairs, or about 4 copies of each template (as supplied in the model kit and shown in Fig. 3.2) is a satisfactory number. The

16 copies should be thoroughly randomized before assembling them into the polynucleotide since a repeating 4 unit structure would be incorrect according to present views. A ring stand may be used to support the final structure. (Note: With 10 pairings each with 4 possibilities the total number of possible arrangements would be 4 to the 10th power or over a million; with 16 it is 4^{16}, cf. discussion on coding in Chapter X).

Each base pair model has four fold-out portions, two corresponding to the deoxyribose moieties and two corresponding to the phosphate moieties, and has two markers indicating an angular displacement of 36° in either direction. The patterns should be cut to permit folding the pentoses to the vertical position; an additional cut on the dotted line around the outline of the furanose ring oxygen will permit it to lie in the vertical plane also. The phosphates should be folded to give alternate horizontal and vertical sections as indicated and extended in the opposite direction from the 3' carbons.

The base pair models should be strung on a vertical wire mounted on the ring stand with each base pair exactly one inch above the previous one using spacers cut from rubber tubing or soda straws and rotated 36° counterclockwise. As each base pair model is added, the 5' phosphate will contact the 3'-OH and can be fastened to it at an appropriate angle. When the final base pair has been added, there will be a 5'-phosphate monoester and a 3'-OH group on each end of the polynucleotide structure, as in the case of the polynucleotides synthesized by the Ochoa and the Kornberg polymerases.

The fully assembled polynucleotide model can be trimmed of excess paper in the areas occupied by the 36° markers if desired. Unpaired nucleotide

models can be prepared by cutting across the hydrogen bonds of a pair and the individual nucleotide models used to study the effects of other pairings. They will be particularly useful for studying the base relationships in triple helix formation, which will be discussed below. The work on the 3-D structure of DNA is continuing and the angular displacement is not finally established as 36°, hence a working model with some flexibility permits ready visualization of current developments.

These hydrogen bondings are not the only ones conceivable but they are the only ones that appear to permit the formation of a double helix that is in agreement with the X-ray diffraction pattern shown by DNA as well as the analytical data and it is generally understood that Watson and Crick developed their theory by attempting to make geometric models fit the analytical and X-ray data.

D.   *Base pairing in RNA*

The analytical data pertaining to base ratios in RNA has not revealed the unique pairings of bases that were found for DNA but they have revealed a regularity in that the ratio of the sum of guanine plus uracil to the sum of adenine plus cytosine is <u>near</u> one. This means that the keto groups on guanine carbon 6 and uracil carbon 4 are equal to the amino groups on adenine carbon 6 and cytosine carbon 4[10]. It may be emphasized that many samples have not shown these regularities but it has been pointed out that degradation of RNA prior to analysis is difficult to avoid[21]. At present no clear explanation of the regularities is available but possible interactions with the -NH- and -CO- group in peptide bonds or with DNA have been considered. At present it is unknown whether the RNA in cells is all single stranded but this is the general conclusion. It is also unknown whether all the RNA carries informa-

tion or whether part of it is storage material. However studies on pairing of RNA strands are now being carried out and may lead to techniques for studying these problems. (see below).

E. *Hydrogen bonding in RNA double and triple helixes*

It was apparent when the Watson-Crick Theory was first proposed that the double helix consisted of two parts of a three part system, that is, the two DNA strands were not symmetrically disposed about the fiber axis but instead were set up in such a way that a groove was available for a third strand. Stent[32] credits Watson and Orgel with the concept that the third strand might be an RNA-protein and that "the specific base sequence of this third RNA would be governed by the base pairs of the DNA duplex, in that each base pair can form another pair of hydrogen bonds, with one and only one type of base, if the C (sugar)-N (base) bond of the third base is to be always in exactly the same position." However there is as yet no certainty as to which bases are determined by the DNA base pairings. Stent has proposed one system and Zubay[36], apparently with the aid of molecular models, has proposed another. The essence of the Zubay proposal is that each base is considered ambivalent in the hydrogen bonding sense and when the DNA double helix takes up a third strand it does so by opening up the hydrogen between pyrimidine 4 and purine 6 in every case. In contrast, Stent's triple helix was assumed to be superimposed on the same bond. The two proposals can be summarized in the following table:

Watson-Crick pair	Stent RNA[32]	Zubay RNA[36]
cytosine-guanine (Fig. 3.3)	cytosine	uracil
guanine-cytosine (Fig. 3.4)	guanine	adenine
thymine-adenine (Fig. 3.5)	uracil	cytosine
adenine-thymine (Fig. 3.6)	adenine	guanine

The hydrogen bonding relationships can be studied best by inserting the single nucleotides into the permitted orientation in a 3-D model as described earlier, in which case it will be observed that the Stent and Zubay RNA strands run in opposite directions.

The work of Ochoa, referred to above[13], provided investigators with long polymers consisting of single bases. This was important because it permitted a direct experimental test of the proposition that an adenine in one chain would form hydrogen bonds with a uracil in an adjacent chain, since if one chain had only adenine and the other chain had only uracil bases, any hydrogen bonding between the two chains could scarcely fail to be defined. Inspection of the adenine-thymine pairing shown in Fig. 3.6 reveals that the groups on the thymine are the -NH- at position 3 and the -CO- at position 4, and these groups are identical with the same positions in the uracil molecule. It might be expected from this that a poly A chain would form a double helix with a poly U, and this has in fact been observed by Warner[34] and by Rich and Davies[28]. Similarly, inspection of Fig. 3.4 reveals that in the guanine-cytosine pair the 1 and 6 positions of guanine are identical with the 1 and 6 positions of hypoxanthine, the base in poly I (for inosinic acid). Since the Ochoa enzyme will produce poly I very successfully from 5'-IDP and poly C from 5'-CDP it has been possible to test for interaction between the two polymers. Davies and Rich[9] have studied the reaction spectrophotometrically. To determine the stoichiometry, the optical density was measured at 235 mμ for a series of mixtures containing varying proportions of poly I and poly C, while keeping the total nucleotide concentration constant. The resulting data show that all mixtures have a lower UV absorption than either polynucleotide alone and yield two straight lines which intersect

sharply at a minimum value corresponding to a 1:1 mole ratio. By the same technique, Felsenfeld, Davies and Rich[11] found that when poly A and poly U were mixed in the absence of magnesium ions the minimum absorption was reached at a 1:1 ratio, but in the presence of magnesium the ratio was 1:2 indicating a triple helix with each A:U pair bonded to another U.

The absorption spectra of nucleic acids had been studied extensively earlier and Chargaff and Zamenhof[6] proposed that the absorption should be expressed in terms of the phosphorus content, since there is one phosphorus atom per nitrogenous base. The molar absorptivity E(P) was accordingly based on one gram-atom of phosphorus per liter. This convention facilitates comparison of UV absorption for nucleic acids before and after hydrolysis or denaturation. It was found that the UV absorption of intact DNA and RNA is always lower than the absorption of the sum of the constituent nucleotides and that various cations decrease the UV absorption of intact DNA. In none of these studies was the relationship a simple one, because the UV absorption appears to be affected by the degree of hydrogen bonding which in turn affects several other features of the structure[2]. These relationships were very difficult to study in highly heterogeneous mixtures of DNA or RNA but are somewhat more meaningful when mixtures of pure poly A and poly U or poly C and poly I are used.

The above discussions of polynucleotide structures provide some foundation for understanding nucleic acid structure in terms of sequences of mononucleotides, the internucleotide bonds, the end groups and the hydrogen bonds between polynucleotide strands. Further details will be brought out in the chapters on biosynthesis and function.

REFERENCES

1. ASTBURY and BELL. Nature 141:747 (1938).
2. BEAVEN, HOLIDAY and JOHNSON in Chargaff and Davidson 1:493 (1954).
3. BROWN and TODD, in Chargaff and Davidson 1:409 (1954).
4. CHARGAFF in Chargaff and Davidson 1:307 (1954).
5. CHARGAFF and LIPSHITZ. J. Am. Chem. Soc. 75:3658 (1953).
6. CHARGAFF and ZAMENHOF. J. Biol. Chem. 173:327 (1948).
7. COHN and VOLKIN. Arch. Biochem. Biophys. 35:465 (1952) J. Biol. Chem. 203:319 (1953).
8. CRESTFIELD and ALLEN. J. Biol. Chem. 219:103 (1956).
9. DAVIES and RICH. J. Am. Chem. Soc. 80:1003 (1958).
10. ELSON and CHARGAFF. Arch. Biochem. Biophys. 17:367 (1955).
11. FELSENFELD, DAVIES and RICH. J. Am. Chem. Soc. 79:2023 (1957).
12. FURBERG. Acta. Chem. Scand. 6:634 (1952).
13. GRUNBERG-MANAGO and OCHOA. J. Am. Chem. Soc. 77:3165 (1955).
14. GULLAND, JORDAN and TAYLOR. J. Chem. Soc. 1947 p. 1131.
15. HEPPEL and HILMOE in Methods in Enzymology II, 565, Academic Press, New York (1955).
16. HEPPEL and RABINOWITZ. Ann. Rev. Biochem. 27:613 (1958).
17. HURST and BUTLER. J. Biol. Chem. 193:91 (1951).
18. JORDAN in Chargaff and Davidson 1:447 (1954).
19. KAPLAN and HEPPEL. J. Biol. Chem. 222:907 (1956).
20. KHORANA in Chargaff and Davidson 3:in press, 1960.
21. MAGASANIK in Chargaff and Davidson 1, 373 (1954).
22. MARKHAM, MATTHEWS and SMITH. Nature 173:537 (1954)
23. PAULING and COREY. Arch. Biochem. and Biophys. 65:164 (1956).
24. PAULING and COREY. Proc. Nat. Acad. Sci. U.S. 39:84 (1953)

25. POTTER, J. L., LAURILA and LASKOWSKI. J. Biol.
      Chem. 233:915 (1958).
26. RAZZELL and KHORANA. J. Biol. Chem. 234:2105,
      2114 (1959).
27. REDDI. Biochim. Biophys. Acta 28:386 (1958).
28. RICH and DAVIES. J. Am. Chem. Soc. 78:3548 (1957).
29. SHUSTER, KHORANA and HEPPEL. Biochim. Biophys.
      Acta 33:452 (1959).
30. SINGER, HEPPEL and HILMOE. Biochim. Biophys.
      Acta 26:198 (1957).
31. SINSHEIMER. J. Mol. Biol. 1:37, 43 (1959).
32. STENT. Advances in Virology 5:95 (1958).
33. TESSMAN. Virology 7, 263 (1959).
34. WARNER. Fed. Proc. 15, 379 (1956) J. Biol. Chem.
      229:711 (1957).
35. WATSON and CRICK. Nature 171:737, 964 (1953) Cold
      Spring Harbor Symp. 18:123 (1953).
36. ZUBAY. Nature 182:112, 388, 1290 (1958).

Chapter IV
Intracellular Localization
of Nucleic Acids

I.   A DEFINITION OF LIFE

II. THE BASIC RELATIONSHIP BETWEEN DNA AND RNA

III. EXPERIMENTAL APPROACHES

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IV. NUCLEIC ACID DISTRIBUTION IN CELL COMPONENTS

    A.   Nuclei
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Chapter IV
Intracellular Localization
of Nucleic Acids

Up to this point we have been mainly concerned with the bare essentials of nucleic acid structure and the structure of the building blocks that combine to form the macromolecules. It seems logical now to establish that the functional relationships between the nucleic acids involve a further organization into more complicated structures that can be regarded as living. At this level, the basic unit of organization is the cell, and any discussion of the function of the nucleic acids must be carried on against a background of information that combines morphological and functional studies.

It would be presumptuous to imply that any limited presentation such as will be outlined below could obviate the need for further studies in formal courses in cytology or for consultation of the many comprehensive articles and books now available. Most of these works make extensive use of high quality reproductions of pictures taken by means of the electron microscope. An appreciation of the elegance of these pictures can only be gained by looking at the highest quality reproductions, if not the originals, and it is fortunate that journals such as the Journal of Biochemical and Biophysical Cytology, which started with volume I in 1955, have undertaken to reproduce such pictures as faithfully as possible. Since presentation of such material is beyond the scope of this book we will emphasize that the material to follow represents a personal point of view and is not intended to supplant the cytological works that are available.

I.   A DEFINITION OF LIFE

During the course of over 20 years this writer has been attempting to spell out an acceptable definition of life in chemical terms and has been revising definitions in the light of current progress in biochemistry. Thinking along this line was stimulated by an essay by N. W. Pirie[33] entitled "The Meaninglessness of the Terms Lifeless and Living." Anyone who has witnessed sudden death is certain to be vaguely dissatisfied by such an essay, and may even begin to suspect that it would be possible to discuss but not to prove the meaninglessness of almost any term expressing such complicated qualities, for example, good and bad. Pirie was of course concerned with the problem of whether viruses are living or lifeless and at this level the question is a legitimate one. Pirie's article in 1938 was prompted by the advent of crystalline viruses and the fact that crystals of virus could maintain "viability" in the crystalline state for extended periods of time, showing no evidence of "life."

Many papers have been written since Pirie's essay, and much knowledge has been gained with very little alteration in the viewpoint usually expressed, namely, that at the border line between viruses and higher forms of life the distinction becomes "meaningless."

However the fact that all viruses are nucleoproteins (Chapter XIII) and the fact that all viable cells contain nucleoproteins leads one to inquire whether the organization of these entities is what may provide a clue to understanding the difference between lifeless and living, and permit us to draw a line between viruses (lifeless) and cells (living).

One of the most intriguing facts in the nucleic acid field is the very existence of two types of nucleic acid, DNA and RNA. There is a growing body of evidence to suggest that viruses consist of one or the other type of

nucleic acid but not both, while viable cells contain both types. Is it not possible that the properties of life are inextricably wrapped up in the relationship between the two major types of nucleic acid? Cells without one or both types of nucleic acids exist but do not possess all the attributes of life. Thus the sperm cell must be considered as a mobile packed of DNA which is not viable in itself but only when it combines with an appropriate egg cell. The odds that a sperm cell will die in the haploid state are certainly better than a million to one, and in any case it will not divide to give two sperm cells.

In an attempt to build a definition of life around the mutual relationship of DNA and RNA the following definition has been developed: (cf. [34]).

"Life consists of the chemical and physical activities of groups of deoxyribonucleoproteins and ribonucleoproteins

operating within organized systems of membranes built of conjugated proteins;

controlling the production of enzymes whose metabolic activities make possible the reproduction of the overall system in a suitable chemical environment by coupling energy-requiring syntheses with reactions that yield energy;

permitting enough variation among individual nucleoproteins to yield a mechanism for evolutionary change by survival of the fittest random variations: and

organized in such a way that changes in the environment set in motion feedback mechanisms altering the production and operation of enzyme systems to give the maximum survival value possible within the limits set by the inherited population of DNA molecules and the existing environment.

The preceding definition escapes from many of the se-
mantic difficulties encountered when attempts to keep
the definition general end up by not excluding mechani-
cal models, or oil droplets, or inorganic crystals or
some other entity easily excluded by the basic assump-
tion that life is the expression of the potentialities of
the nucleic acids, and that lifeless nucleic acids can
evolve into living organisms with potential further evo-
lution.

II.   THE BASIC RELATIONSHIP BETWEEN DNA AND RNA

The definition of life presented in the last section omits
the details of how the results are attained, and it is now
our task to identify some of the specific roles of the two
types of nucleic acids.

The basic relationship appears to be one which para-
phrases Samuel Butler's statement that a hen is only
an egg's way of making another egg: RNA appears to
be DNA's way of making more DNA. When we speak of
self-reproducing molecules of DNA we mean self-repro-
ducing in a milieu of the proper building blocks and en-
zymes and this has always to be understood. The term,
self-reproducing, is meaningful in that a DNA molecule
is reproduced only in the presence of the same mole-
cule, which serves as a template for its own reproduc-
tion. This, of course, is not the case for smaller
molecules, which are always produced from one or
more precursors usually by the action of an enzyme.
Thus the DNA is a self-reproducing molecule in the
right milieu: the DNA molecules in living systems in-
sure the milieu by making RNA molecules, which in
turn provide the right enzymes and small molecules to
permit DNA synthesis. Now that we know what building
blocks and enzymes are needed, it is possible to get
DNA synthesis in non-living systems (Chapter VII).

According to the best available knowledge the DNA mol-
ecules correspond to one or more Mendelian genes
(Chapter X) and have a two-fold function: the replica-
tion of themselves and the transmission of the molecu-
lar patterns of the proteins that go to make up the
enzymes and structural units of the cell. The DNA mol-
ecules apparently do not act directly in all structural
and enzyme proteins and it has yet to be proved that
they are directly concerned in the synthesis of any.
Protein synthesis appears to be the special function of
the RNA molecules, and the latter apparently carry out
the synthesis according to patterns received from the
DNA molecules.

The above paragraph suggests a picture that is consider-
ably simpler than actual practice. Although DNA is
located in chromosomes and behaves according to what
might be expected of a Mendelian gene, there are many
kinds of RNA if classified according to intracellular
location, and although the suggested functional role is
supported by available data, there are many unanswered
questions. For example, does the DNA function continu-
ously in producing RNA molecules or are the latter
formed only at cell division? Does the number of RNA
molecules that replicate gene patterns bear a fixed
numerical ratio to the latter? Do some RNA molecules
get replicated more often than others? Are all RNA
molecules replicates of DNA patterns or are some RNA
molecules independently replicating?

In any case, recent knowledge of a class of RNA mole-
cules of low molecular weight, i.e., about 15-20,000,
has revealed that this kind of RNA has the general
properties of the coenzymes in that they appear to have
acceptor-donor functions for the amino acids, with each
amino acid transferred by a specific type of RNA mole-
cule. This class of RNA is now referred to as soluble
RNA (S-RNA) or transfer-RNA[18] although it is by no
means clear that all S-RNA as obtained by centrifugal
techniques is in fact functional as transfer-RNA.

The bulk of the RNA in the cells is of high molecular weight, particulate in nature, and at present closely identified with the function originally assumed for RNA in general, namely the EFS, or enzyme-forming system, which has been assumed to carry the pattern from the DNA molecules and to use it for the synthesis of specific proteins. Here we are referring to the RNA of the particles now called ribosomes and found in the cytoplasm. These two types of RNA have been thought to work together, the transfer-RNA bringing activated amino acids to the EFS and unloading them in sequences determined by matching a code that is common to the transfer-RNA and the EFS.[18] This interpretation assumes a fairly direct relationship between the DNA and the RNA of the ribosomes and a more indirect relationship between the DNA and the transfer-RNA. Since the latter has an essentially coenzymic function, the relationship to DNA need be no more intimate than for any other coenzyme, that is, given gene patterns are more likely to be for the enzymes that synthesize the coenzyme and participate in acceptor-donor functions with it than for the synthesis of the coenzyme directly.

The additional sites of RNA localization in cells are in the nuclei, in both the chromosomes and the nucleoli, and in mitochondria. The RNA in these locales may be considered analogous to either the ribosomal or the transfer-RNA or may be evidences of interaction with DNA and be enroute from the site of the latter to the cell compartment in which the RNA function is carried out. Current studies are very definitely based on a knowledge of cell architecture and assume that considerable freedom of movement from one cell compartment to another is possible, although this movement must be subject to exquisite controls.

III.   EXPERIMENTAL APPROACHES

The recent gains in the integration of biochemistry and cytology have come from essentially two types of ex-

perimental study, the microscopic or visual methods, and the cell fractionation or centrifugal techniques. The relative roles have been admirably presented by Hogeboom and Schneider.[19]

A. *Microscopic techniques*

The classic approach, dating back to Virchow at least, has as its modern representatives men who have linked staining techniques to biochemistry. Brachet[3, 4] and Davidson[9] pointed out the parallelism between the basophilia of various tissues (affinity for stains such as hemotoxylin) and the RNA content. A variant of the stain technique has been pioneered by Caspersson[7], who micro-photographed histological sections with the help of monochromatic ultraviolet light in order to localize nucleotides and protein. Throughout this period and continuing to the present, the specific staining of DNA structures by the Feulgen technique has remained a fixed feature since 1924 and has been refined to highly quantitative terms.[42] With the advent of radioactive precursors, the autoradiograph has been combined with the staining techniques. Sections on glass slides are stored in contact with photographic emulsions and when these are developed the radioactive precursors can be localized within individual cells, as shown in early studies by Howard and Pelc,[22] Ficq,[13] and Taylor.[43] See review by Ficq.[14] The advent of tritium-labeled thymidine has greatly multiplied the number of investigators using autoradiographic techniques for the study of DNA synthesis.[41] Most of the above studies have been carried out on the acid insoluble components of cells, but a recent paper by Bell[2] reports on the localization of a diffusible nucleotide in frozen-dried sections.

With the advent of the electron microscope and the combination of cell fractionation techniques with

this powerful tool, the way was opened for an integration and interpretation of chemical data obtained on cell fractions with the morphological structures seen at high magnification in thin sections as exemplified by the elegant studies reported by Palade.[28]

B. *Cell Fractionation*

The techniques of cell fractionation, pioneered by Claude, have played an extremely important role in the understanding of nucleic acid metabolism. Using the homogenate technique[36] as a starting point, Schneider and Potter began a program that was described in terms of cell fractionation, enzyme assays, and nucleic acid determinations in the following terms[35]: "The testing of the concepts.. ...requires the combination of two highly technical procedures: (a) the biological and histological (cytological) identification of the particles, and their separation from the other components of the cell without the loss of catalytic potency; and (b) the qualitative and quantitative measurement of the nucleic acid content and of the specific enzymatic activities of the various types of particles."

Studies combining enzyme assays and nucleic acid determinations on cell fractions were first carried out by Schneider[39] whose experience was fortunately later combined with that of Hogeboom and Palade in Claude's laboratory at the Rockefeller Institute to devise improved centrifugal techniques and cytological identification of isolated mitochondria[20, 21]. Hogeboom and Schneider later collaborated on the study of cell fractions at the National Cancer Institute (cf[19]) while Palade[28, 29, 30] and Palade and Siekevitz[31, 32] concentrated on the resolution of the microsome fraction by means of an integrated study in which the electron microscope was applied to thin tissue sections and to cell fractions prepared from homogenates of the same tissues. These

classic studies have established the identity of the small particles[29], now referred to as ribosomes, in relation to the membranous plates that are referred to as the endoplasmic reticulum[30] and established the fact that they account for the bulk of the ribonucleic acid in the cell in the form of ribonucleoprotein[18, 31, 32]. The foregoing studies have been particularly important in establishing a solid cytological foundation for the present surge of activity which has established the importance of the ribosomes and soluble RNA in protein synthesis.[18] Largely as a result of the studies by Palade and Siekevitz[31, 32] we now know that the process of "homogenization" disrupts the membranes of the endoplasmic reticulum and pinches them off into small elements that are capable of acting as osmometers, imbibing water in hypotonic solution. These vesicles of random size, with ribosomes attached to their outer walls, constitute what has been generally referred to as the "microsome fraction". Thus this fraction is actually an artefact which is nevertheless capable of carrying out many biochemical reactions that have hitherto occurred only in intact cells[18, 40]. The membrane portion contains phospholipids and proteins[31] and its role in the function of the ribosomes is still not clear.

IV.   NUCLEIC ACID DISTRIBUTION IN CELL COMPONENTS

The cell fractions obtained by centrifugal techniques vary according to the dispersion media and the speeds employed. Although much remains to be done in separating cleaner fractions, the fact that the larger particles in the microsome fraction seem to be artifacts makes it appear that there is some justification for using the centrifugal separations as approximations separating the major components, and carrying on further separations by chemical means. Moreover, even in a tissue such as rat liver which has been so widely em-

ployed, it must be remembered that although nearly all
of the mitochondria come from parenchymal liver cells,
only about 60% of the nuclei do. [10]

Some brief remarks on specific cell fractions may be
given with the above limitations in mind.

A.   *Nuclei*

In the case of rat liver homogenates in 0.25 M su-
crose the nuclei can be separated at 600g (600x
gravity) for 10 minutes. Nuclei from tumor homo-
genates have been purified further by means of
hypertonic sucrose and higher speeds according to
the technique of Busch et al. [5].

Cell nuclei appear to contain all of the DNA of the
cell, which in general is concentrated in the chro-
mosomes[44] which can be isolated by the centrifugal
techniques[38]. Such preparations have been used for
the isolation of DNA protein believed to be in native
form. [48] It has recently been possible to demonstrate
the localization of DNA in $\underline{E}$. $\underline{coli}$[6]. The DNA con-
tent of bacteria (one species: $\underline{Clostridium}$ $\underline{Welchii}$)
is only about 1/200 the amount found in the nuclei
of animal cells which averages between 5.0 and 6.0
picograms (grams x 10^{-12}) for a number of species.[47]
It seems unlikely that animal cells contain 200
times as many kinds of enzymes as bacterial cells,
and the data suggest that perhaps each gene is
represented more times in the higher forms. Some
calculations related to this question are as follows:
Assume 6 x 10^{-12} grams DNA per human cell and
8 x 10^6 as the average molecular weight. Avogadro
number = 6.02 x 10^{23}; then (6 x 10^{-12}) (0.125 x 10^{-6})
(6 x 10^{23}) = 450,000 molecules of DNA per human
diploid cell. Assume average molecular weight of
1 nucleotide pair = 618 and internucleotide distance
= 3.4 Angstrom units = 3.4 x 10^{-10} meters. Then
(8 x 10^6) (6.18 x 10^{-2}) = 12,900 nucleotide pairs per

DNA molecule, or 5.8 billion nucleotide pairs per cell. Total length = (5.8×10^9) $(3.4 \times 10^{-10}$ meters) = 1.97 meters or 6.45 feet of double helix per cell. In a similar way it could be calculated that the bacterial cells referred to would contain approximately 2250 molecules of DNA. A recent publication describes what may be the simplest form of life recorded up to this time, with an amount of DNA so small that it could provide for not more than 8 or 9 molecules of such DNA[27].

Dounce[11] has reviewed the literature on the DNA and RNA content of nuclei. In rat livers the DNA content per organ shows very little change in adults during starvation and in fact is extremely constant on a per cell basis, within a given species, in normal resting diploid somatic cells, and is one-half the diploid values in spermatozoa. The per cent of DNA in nuclei varies from 4 to 26 per cent of the dry weight, depending on tissue, diet, and procedure, and reflects variation in non-DNA constituents, chiefly protein. The RNA content of nuclei, like the protein, is quite variable. The bulk of the nuclear RNA is localized in the nucleoli but isolated chromosomes also contain RNA.[26] Nucleoli have been isolated and reported to contain DNA in addition to RNA but the relationship between the two types is not clear. That the RNA in chromatin and in the nucleoli are metabolically distinct has recently been demonstrated by autoradiographic[15,24,25] and other techniques.

B. *Mitochondria*

The mitochondria of rat liver are readily isolated from the supernatant fraction (S_1) obtained from the nuclear fraction, and may be isolated at centrifugal forces of about 5000xg or less. Even at this speed there is contamination from the RNA-rich microsome fraction and at higher speeds the contamination increases as shown by Potter et al.[37]. There

has been considerable doubt as to whether mito-
chondria contain RNA at all but the metabolic data
of Hecht and Potter[17] show that this RNA fraction
has a significantly lower specific activity than any
other fraction. Data obtained by Simpson clearly
demonstrate protein synthesis in mitochondria, and
this was sensitive to RNAse in sonicates but not in
whole mitochondria (quoted by Hoagland.[18])

C.   *Microsome Fraction*

As indicated earlier, the microsome fraction is de-
rived from the endoplasmic reticulum[29] plus its at-
tached ribonucleoprotein particles[30] or ribosomes.
Although the microsome fraction is usually collect-
ed by centrifuging the supernatant from the mito-
chondrial fraction (S_2) at forces of 105,000 xg for
about 60 minutes, the yield of RNA protein contin-
ues to increase with prolonged centrifugation. This
has less effect percentagewise on the amount of the
microsomal RNA recovered than it does on the
amount of soluble RNA since the latter is a much
smaller fraction. It appears that the microsome
fraction sediments at 60,000 xg within less than
one hour, while the higher gravitational force and
longer time periods are required to sediment the
free or unattached ribosomes in a "post-microso-
mal" fraction. The proportion of RNA in the two
microsomal fractions is apparently different in
liver[31] and pancreas[32] and may vary with physiolog-
ical state.

The ribosomes are fairly uniform in size and aver-
age 100-200 Angstrom units in diameter. The mol-
ecular weight of the RNA is about 2.0×10^6 but
there may be sub-units that are as small as 250,000.
The total microsome fraction accounts for as much
as 80-90% of the RNA in the liver cell[18], and 50-
65% of the RNA in the pea seedling,[45] and in pan-
creas.[46]

D.   *Soluble RNA* [18]

At present the amount of soluble RNA is arbitrarily
based on the fraction that fails to sediment at
105,000g applied for several hours.   The soluble
RNA appears to be free of protein but is largely
combined with amino acids attached at the 3' end
of each RNA molecule.   The fraction is said to
comprise 10-20% of the total RNA of the cell, and
apparently is a mixture of transfer-RNA molecules
in which each amino acid is combined with a differ-
ent species of RNA.   At present it is unclear wheth-
er all of the RNA in this fraction is involved in
amino acid transfer.   As of the present, the frac-
tion is unique in containing unusual purines and
pyrimidines in considerable amounts.   It remains
to be seen whether the RNA molecules that contain
unusual bases are also involved in amino acid
transfer.

E.   *Other cell constituents*

There are many other features of the cell that are
known to the cytologist but are either not available
by centrifugal techniques or are not characterized
in terms of nucleic acid content or function.   These
include the nuclear and cell membranes, the Golgi
apparatus, the lysosomes and mitotic apparatus
which will not be discussed at this point.   Further
material on these and fractions already mentioned
may be found in the references cited. [1, 8, 12, 16, 19, 23]
It may be emphasized that the newer knowledge re-
garding soluble RNA[18] calls for a reexamination of
most of the statements that were made before this
fraction was treated separately.   Similarly, many
studies involving fractionation of cytoplasmic RNA
were made before the nature of the microsome
fraction was understood.   Much of our progress
can be traced to the studies in which the electron
microscope was used to study the fractions obtained

from tissue homogenates[31, 32] and these studies emphasize the need for using both biochemical and cytological approaches in the study of the function of the nucleic acids. If these approaches cannot be combined in the same laboratory we must at least try to note some of the ways in which results in different laboratories are related.

REFERENCES

1. *ALLFREY in Brachet and Mirsky. The Cell, Vol. I, Academic Press, (1959).*
2. *BELL. J. Histochem. Cytochem. 6:435 (1958).*
3. *BRACHET. Enzymologia 10, 87 (1941).*
4. *BRACHET, in Chargaff and Davidson. 2:475 (1955).*
5. *BUSCH, STARBUCK and DAVIS. Cancer Research 19:684 (1959).*
6. *CARO, van TUBERGEN, and FORRO. J. Biophys. Biochem. Cytol. 4:491 (1958).*
7. *CASPERSSON. Symp. Soc. Exptl. Biol. 1, 127 (1947).*
8. *CROOK. The Structure and Function of Subcellular Components. Cambridge University Press, New York (1959).*
9. *DAVIDSON. Cold Spring Harbor Symposia Quart. Biol. 12, 50 (1947).*
10. *DAOUST and CANTERO. Cancer Research 19:757 (1959).*
11. *DOUNCE in Chargaff and Davidson. 2:93 (1955).*
12. *DUVE, PRESSMAN, GIANETTO, WATTIAUX and APPELMANS. Biochem J. 60, 604 (1955).*
13. *FICQ. Experentia 9, 377 (1953).*
14. *FICQ in Brachet and Mirsky. The Cell, Vol. 1, Academic Press (1959) New York.*
15. *GOLDSTEIN and MICOU. J. Biophys. Biochem. Cytol. 6:301 (1959).*
16. *HAYASHI. Subcellular Particles, Ronald Press, New York (1959).*
17. *HECHT and POTTER. Cancer Research 18:186 (1958).*
18. *HOAGLAND in Chargaff and Davidson. 3:in press (1960).*
19. *HOGEBOOM and SCHNEIDER in Chargaff and Davidson. 2:199 (1955).*

20. HOGEBOOM, SCHNEIDER and PALADE. Proc. Soc. Exp. Biol. Med. 65:320 (1947).

21. HOGEBOOM, SCHNEIDER and PALADE. J. Biol. Chem. 172, 619 (1948).

22. HOWARD and PELC in J. N. Davidson, Isotopes in Biochemistry, Churchill, London (1951).

23. KUFF and HOGEBOOM in Gaebler, Enzymes: Units of Biological Structure and Function. Academic Press, New York (1956).

24. McMASTER-KAYE and TAYLOR. J. Biophys. Biochem. Cytol. 4:5 (1958).

25. McMASTER-KAYE and TAYLOR. J. Biophys. Biochem. Cytol. 5:461 (1959).

26. MIRSKY. Cold Spring Harbor Symposia Quart. Biol. 12:143 (1947).

27. MOROWITZ and CLEVERDON. Biochim. Biophys. Acta 34:578 (1959).

28. PALADE in Gaebler, Enzymes: Units of Biological Structure and Function. Academic Press, New York (1956).

29. PALADE. J. Biophys. Biochem. Cytol. 1:59 (1956).

30. PALADE. J. Biophys. Biochem. Cytol. 2:85 (1956).

31. PALADE and SIEKEVITZ. J. Biophys. Biochem. Cytol. 2:171 (1956).

32. PALADE and SIEKEVITZ. J. Biophys. and Biochem. Cytol. 2:671 (1956).

33. PIRIE in Green and Needham. Perspectives in Biochemistry (1938).

34. POTTER. U. Mich. Med. Bull. 23:401 (1957).

35. POTTER in Werkman and Nord, Advances in Enzymology Vol. 4, Interscience, New York (1944).

36. POTTER and ELVEHJEM. J. Biol. Chem. 114:495 (1936).

37. POTTER, RECKNAGEL and HURLBERT. Fed. Proc. 10:646 (1951).

38. RIS and MIRSKY. Exp. Cell Research 2:263 (1951)

39. SCHNEIDER. J. Biol. Chem. (1945).

40. SIEKEVITZ and PALADE. J. Biophys. Biochem. Cytol. 5:1 (1959).

41. STOHLMAN. *The Kinetics of Cellular Proliferation* Greene and Stratton, New York (1959).
42. SWIFT, in Chargaff and Davidson. 2:51 (1955).
43. TAYLOR. Science 118, 555 (1953).
44. THORELL in Chargaff and Davidson. 2:181 (1955).
45. TSO and SATO. Exp. Cell Research 17:227 (1959).
46. VAN LANCKER and HOLTZER. J. Biol. Chem. 234:2359 (1959).
47. VENDRELY in Chargaff and Davidson. 2:155 (1959).
48. ZUBAY and DOTY. J. Mol. Biol. 1:1 (1959).

Chapter V
The Biosynthesis of the
Sugar Moieties

Chapter V
The Biosynthesis of the
Sugar Moieties

I. INTRODUCTION

The biosynthesis of the sugar moieties of the nucleic
acids will be discussed at this point in order to lay the
foundation for the next chapter, which deals with the
biosynthesis of the purines and pyrimidines. This se-
quence is logical because it will be shown that the
ribose moiety is built up to the level of an "energized"
building block in which ribose-5-phosphate carries a
pyrophosphate group on the No. 1 carbon. This pyro-
phosphate is then replaced by the no. 1 nitrogen of the
pyrimidines or the no. 9 nitrogen of the purines or by
the nitrogen of compounds that are finally built up to
pyrimidines or purines. Attempts to find an analogous
phosphodeoxyribosylpyrophosphate have been unsuccess-
ful, and it appears that the deoxyribosides can be form-
ed either by the reduction of certain ribosides or ribo-
tides or by the exchange of free bases with the phosphate
of deoxyribose-1-phosphate, a reaction that is analogous
to the similar reaction between the bases and ribose-1-
phosphate. The present chapter will accordingly deal
with the synthesis of five intermediates: (1) ribose-5-
phosphate, (2) 5-phosphoryl- -D-ribofuranose-1-pyro-
phosphate, PRPP, (3) ribose-1-phosphate, (4) 2-deoxy-
ribose-1-phosphate, and (5) 5'-deoxyribotides. In all
cases, glucose and derivatives of glucose appear to be
the most important distal precursors, with exceptions
in the case of particular microorganisms.

II.   HISTORICAL

The above outline of the biosynthesis of the sugar moieties of the nucleic acids is greatly simplified by the fact that up to this time the only sugars found in the nucleic acids have been D-ribose and 2-deoxy-D-ribose. Although in 1959 Smith and Dunn[44] reported the isolation of small amounts of dinucleotides from alkaline hydrolysates of RNA from various sources and reported that they contained sugar moieties that are neither ribose nor deoxyribose, it appears most probable that these unusual nucleotides contain ribose with a methyl group on the 2'OH. This would account for the appearance of the dinucleotides in the alkaline hydrolysates, since the 2'OH has to be available for formation of a 2'3' cyclic phosphate intermediate during hydrolysis. Although the new dinucleotides yielded nucleosides that resembled 2'-O-methyl ribosides that were available for comparison, further work may reveal that the substituent is not a methyl group or that other substituents can also occur at the 2' position. Examination of the polynucleotide structure (Chapter III) will reveal that such substituents would not be incompatible with the 3'5' phosphate diester linkages that form the basis of the polynucleotide backbone.

In trying to explain why the sugars that form the basis of the nucleic acid structure are always D-ribose or a minor modification of it (viz. 2'deoxy-D-ribose or 2'-O-methyl-D-ribose) it is instructive to study a 3-dimensional model of a double helix (Chapter III) and to note that the orientation and availability of the 3' and 5' OH groups is an important feature of the structure. The 2' position is not obviously implicated in the 3 dimensional structure but does have important consequences from a functional standpoint. While a totally different kind of nucleic acid might be imagined, we are forced to the conclusion that either the first nucleic acid to be formed spontaneously determined the character of all its successors, or that the ribose and deoxyribose nucleic acids had unusual competitive superiority over the other chance beginners.

The occurrence of carbohydrate in ribonucleic acid was demonstrated in 1891 by Kossel and three years later Hammarsten[17] showed that the sugar was a pentose. The actual identification of the pentose as D-ribose by Levene and Jacobs in 1909[24] and the final confirmation by synthesis in 1913 has been reviewed by Overend and Stacey[30].

The identification of the deoxyribose in DNA was considerably more difficult and for many years Levene and others believed that the sugar in DNA was an aldohexose because the acid hydrolysis of DNA leads to the formation of levulinic acid, CH_3COCH_2COOH, which is also formed from aldohexoses via hydroxymethylfurfural
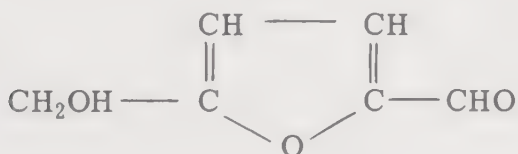
In the case of 2-deoxy-D-ribose under conditions of acid hydrolysis the precursor of levulinic acid is omega-hydroxy-levulinaldehyde, $CH_2OHCOCH_2CH_2CHO$, which reacts with diphenylamine in the Dische test to give a blue color that is used as the basis of a quantitative analytical method. The final identification of 2-deoxy-D-ribose depended upon enzymic methods of hydrolysis, and was achieved by Levene, Mikeska, and Mori in 1930[25].

The biosynthesis of these sugars was not a matter of investigation until after the Embden-Meyerhof scheme of anaerobic glycolysis was worked out in 1933-1939. During this period Warburg showed the existence of a pathway of glucose breakdown that was separate from the glycolytic pathway. It was oxidative in nature and led to the formation of 6-phosphogluconic acid. The further breakdown of this substance to a pentose was studied by Dickens and by Warburg but positive identifi-

cation of the pentose was not accomplished at this time.[13] In a 1944 review of the data on this pathway, referred to as the hexosemonophosphate shunt or "HMP shunt", Potter[32] commented that "Dickens' suggestion that ribose-5-phosphate is an intermediate in this pathway would be expected to assume great importance in growing tissues in which ribose-5-phosphate would be needed as a building block for the synthesis of ribonucleoproteins..."

Actual investigation of the biosynthesis of the pentoses got underway in 1950 when S. S. Cohen and D. B. M. Scott, and Horecker and Smyrniotis undertook to reinvestigate the HMP shunt and to identify the pentoses.[13] It was shown that a key intermediate is ribulose-5-phosphate, which is the keto-sugar isomer of ribose-5-phosphate. This compound was shown to be formed from 6-phosphogluconic acid by oxidation with TPN as a coenzyme and to be converted to ribose-5-phosphate with both yeast and liver[43] enzymes.

Meanwhile a large number of investigations pointed to a series of non-oxidative reactions by which pentose phosphates could be converted to triose phosphate and to hexosemonophosphate. These reactions were shown to be reversible and to involve the intermediate formation of both 4- and 7-carbon intermediates. At the time the subject was reviewed in 1954 by Glock[13], in 1954 by Racker[35] and in 1955 by Horecker and Mehler[18] the picture was nearly complete, but it remained for a memorable series of papers by Horecker and coworkers in 1956[19] to complete the connection between the HMP shunt and the non-oxidative two- and three-carbon shifts by establishing xylulose-5-phosphate as the intermediate between ribulose-5-phosphate and the 2, 3, 4, 6, and 7 carbon intermediates, and the identification of α-5-phosphoribosylpyrophosphate by Kornberg et al[23] in 1954 and 1955 to complete the connection between all of these reactions and the ribose of the nucleotides.

The net effect of the coexistence of the oxidative HMP shunt and the non-oxidative two- and three-carbon shifts is that several alternative pathways exist for the pro-

duction and reutilization of ribose-5-phosphate, and all of the current studies seem to indicate that in almost no instance is one pathway used to the total exclusion of the others. The factors that control the balance between the various pathways in different organisms and tissues may occupy the attention of biochemists for some time to come.[7]

The available information on the remaining intermediates will not be discussed in this section because the perspective is not adequately developed. The steps leading to the individual compounds mentioned in the introduction will now be described.

III. RIBOSE-5-PHOSPHATE

A. Oxidative Formation

The following reactions lead from glucose to ribose-5-phosphate, with the sugars written in aldehyde or keto forms:
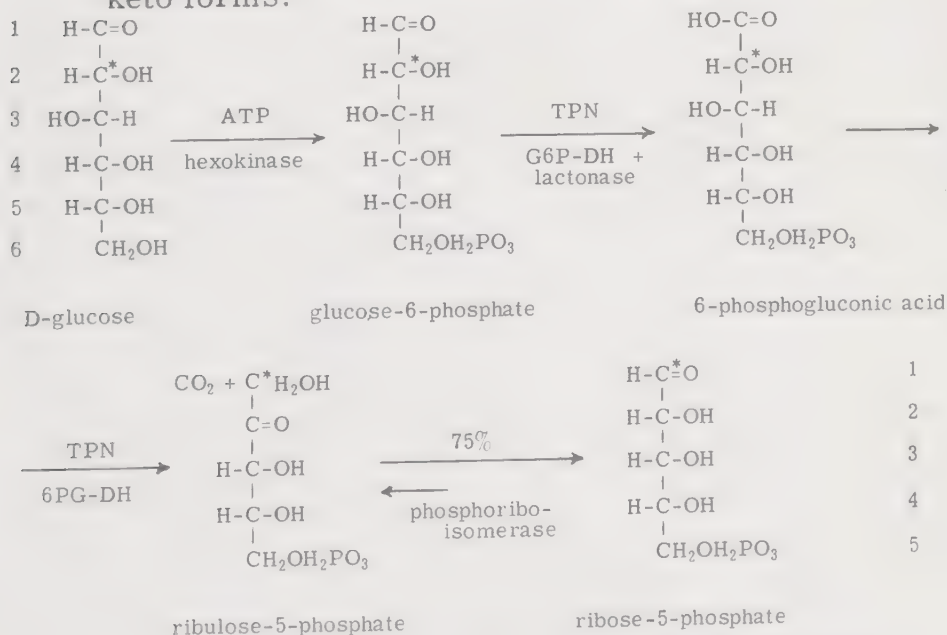
Fig. 5.1. Oxidative formation of ribose-5-phosphate from glucose and glucose-6-phosphate. DH used as the abbreviation for dehydrogenase. An asterisk is used to indicate the position of a C^{14} carbon atom introduced in glucose-2-C^{14}. The numbering of the hexose and pentose carbons is indicated at the beginning and end of the series, respectively.

Branches off the above pathway have been reported at the level of glucose-6-phosphate, ribulose-5-phosphate, and ribose-5-phosphate. The conversions of glucose-6-phosphate to glucose-1-phosphate and fructose-6-phosphate are well known. The conversion of ribulose-5-phosphate to xylulose-5-phosphate[19, 45] connects the <u>oxidative</u> scheme to the <u>non-oxidative</u> network:
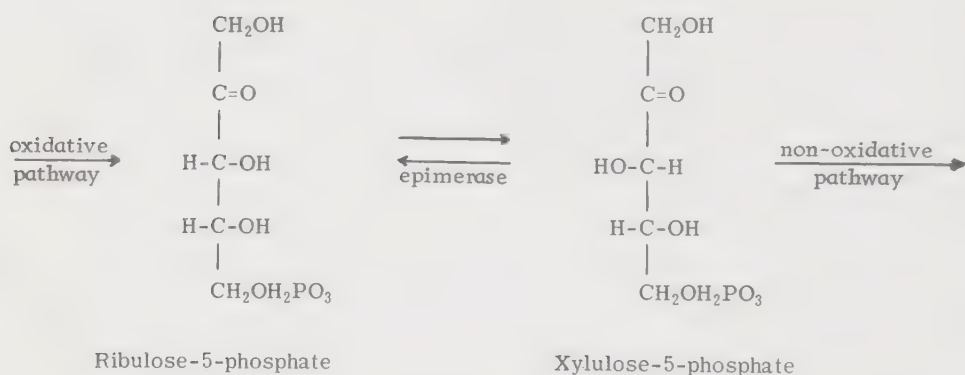
Fig. 5.2   The epimerase connection between the non-oxidative pathway and the oxidative pathway of ribose-5-phosphate synthesis

The cleavage of xylulose-5-phosphate to triose phosphate and a C_2 fragment by transketolase provides a source of C_2 which can condense with ribose-5-phosphate to form sedoheptulose-7-phosphate, the second connection between the oxidative and the non-oxidative scheme. Thus two moles of C_5 can yield one mole each of C_3 and C_7, in keeping with the results of Horecker and Smyrniotis, if other enzymes are excluded.[20] The natural occurrence of sedoheptulose-7-phosphate in liver has been shown.[29]

In addition to the above branches, we have to bear in mind that <u>outgoing</u> branches from those of our immediate interest may be found. Thus ribulose-5-phosphate can be converted to ribulose-1:5-diphosphate and this can pick up CO_2 in photosynthetic systems and cleave to form two moles of phosphoglyceric acid.[19]

B. Transketolase Reactions (Coenzyme: Thiamine-pyrophosphate)

The transketolase reactions involve the activation of a terminal C_2 from the non-phosphorylated end of a homologous series of C_5, C_6, and C_7 keto-sugar phosphates that include fructose-6-phosphate.[35, 36] The following reactions show the analogies in this series.

```
   CH2OH                              CH2OH
    |                                  |
   C=O                                C=O
----+--------                          |
HO-C-H                               Ez-H        +        H-C=O
    |                          →                            |
  H-C-OH      + Ez             ⇄                          H-C-OH
    |                                                       |
  H-C-OH                                                  H-C-OH
    |                                                       |
  H-C-OH                                                  H-C-OH
    |                                                       |
   CH2OH2PO3                                             CH2OH2PO3
```

Sedoheptulose-7-phosphate   Ribose-5-phosphate

```
   CH2OH                              CH2OH
    |                                  |
   C=O                                C=O
----+---------                         |
HO-C-H                               Ez-H        +        H-C=O
    |                          →                            |
  H-C-OH      + Ez             ⇄                          H-C-OH
    |                                                       |
  H-C-OH                                                  H-C-OH
    |                                                       |
   CH2OH2PO3                                             CH2OH2PO3
```

Fructose-6-phosphate   Erythrose-4-phosphate

```
   CH2OH                              CH2OH
    |                                  |
   C=O                                C=O
----+---------                         |
HO-C-H                               Ez-H        +        H-C=O
    |                          →                            |
  H-C-OH      + Ez             ⇄                          H-C-OH
    |                                                       |
   CH2OH2PO3                                             CH2OH2PO3
```

Xylulose-5-phosphate   Glyceraldehyde-3-phosphate
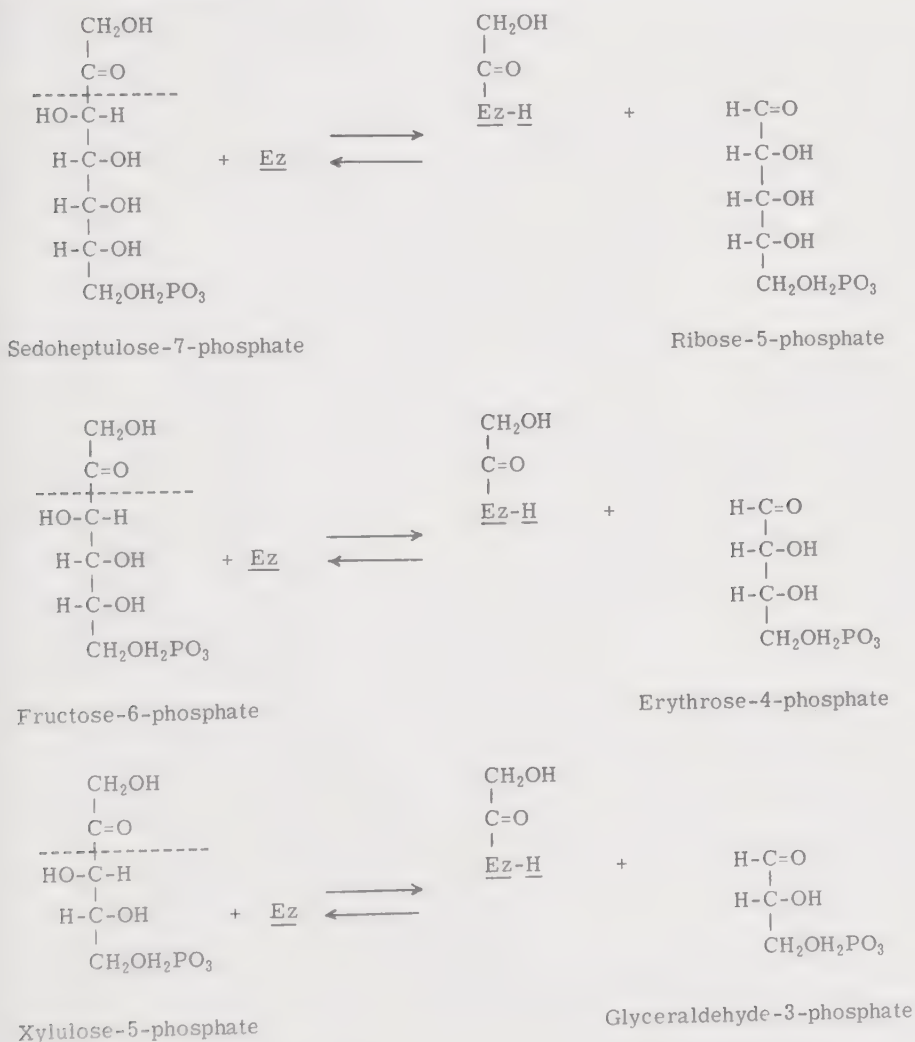
Fig. 5.3. Transketolase reactions in the non-oxidative pathways between fructose-6-phosphate and ribose-5-phosphate. The dashed lines show the points of cleavage. Note identical structures above the lines and the homologous series below the lines.

In the preceding reactions the C_2 fragment has been written in combination with the enzyme to show that it does not occur in the free state. When the trans-ketolase or C_2 transfers occur in the presence of transaldolase reactions involving the same substrates, the result offers considerable opportunity for recombining C_2 and C_3 fragments. The transaldolase and aldolase reactions are shown below.

C.   *Transaldolase and Aldolase Reactions*

The transaldolase reactions involve the activation of a terminal C_3 from the non-phosphorylated end of sedoheptulose-7-phosphate and fructose-6-phosphate:


```
    CH2OH                              CH2OH
    |                                  |
    C=O                                C=O
    |                                  |
 HO-C-H                             HO-C-H
--------+-------                        |
    H-C-OH      +   Ez   ⇌            Ez-H    +    H-C=O
    |                                             |
    H-C-OH                                      H-C-OH
    |                                             |
    H-C-OH                                      H-C-OH
    |                                             |
    CH2OH2PO3                                   CH2OH2PO3
```

Sedoheptulose-7-phosphate   Erythrose-4-phosphate

```
    CH2OH                              CH2OH
    |                                  |
    C=O                                C=O
    |                                  |
 HO-C-H                             HO-C-H
--------+---------                      |
    H-C-OH      +   Ez   ⇌            Ez-H    +    H-C=O
    |                                             |
    H-C-OH                                      H-C-OH
    |                                             |
    CH2OH2PO3                                   CH2OH2PO3
```

Fructose-6-phosphate   Glyceraldehyde-3-phosphate
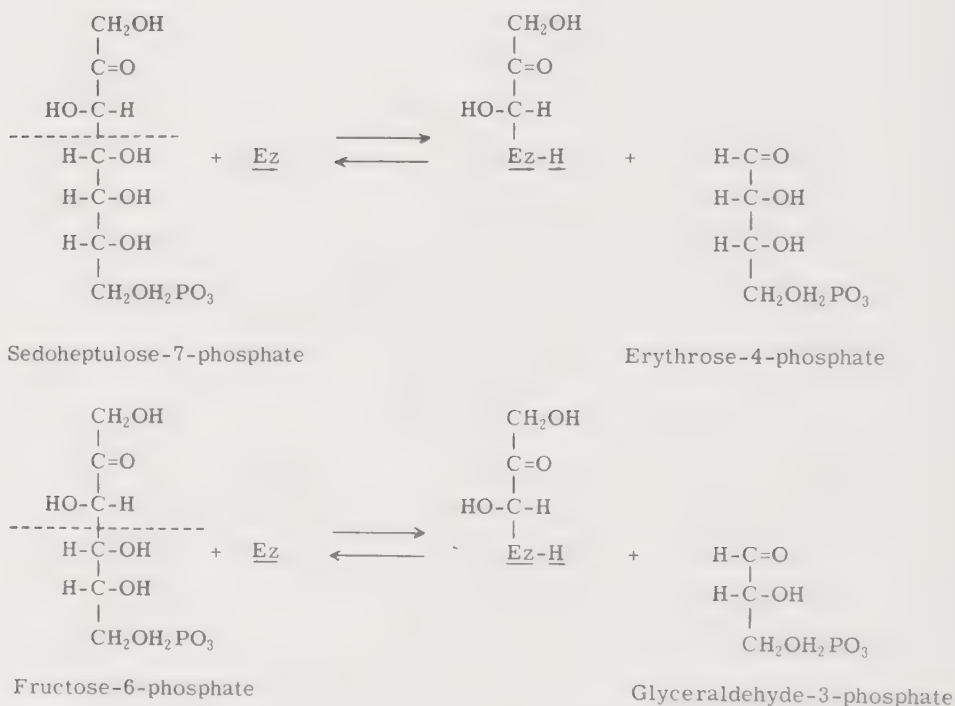
Fig. 5.4. Transaldolase reactions in the non-oxidative pathway between fructose-6-phosphate and ribose-5-phosphate. The dashed lines show the points of cleavage as in Fig. 5.3.

In addition to the preceding reactions there are aldolase reactions in which the fragments appear in free form. These include the well known cleavage of hexosediphosphate:
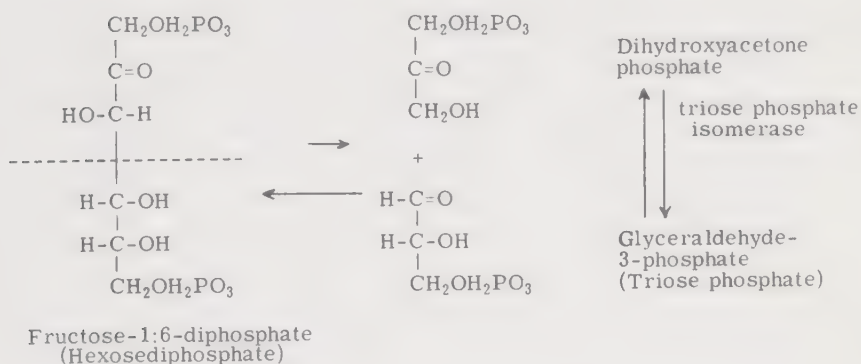
Fig. 5.5. Cleavage of hexosediphosphate by aldolase

This reaction is an essential step in glycolysis, and the quantitative importance of this reaction is such that it is always available to take up or give off triosephosphate to balance any reactions involving the pentose phosphates. Some examples of such reactions are given in the next section.

D. Balanced Reaction Sequences

There are four enzymes in the oxidative pathway from glucose-6-phosphate to ribose-5-phosphate (Fig. 5.1), and the pentoses are connected to the Embden-Meyerhof pathway by 3 more enzymes, namely the epimerase (Fig. 5.2) that interconverts ribulose- and xylulose-phosphates, transketolase (Fig. 5.3), and transaldolase (Fig. 5.4). The reactions that are possible may be considered as oxidative, non-oxidative, or mixed, and they can be considered as converting hexose phosphates to ribose phosphates, while the reverse reactions are entirely non-oxidative.

1. Oxidative Pathway. The oxidative pathway in-
    dicated in Fig. 5.1 is very simple from a
    stoichiometric standpoint. Starting with glu-
    cose-6-phosphate, the overall reaction will be:

    glucose-6-phosphate → ribose-5-phosphate + CO_2

    and if the glucose-6-phosphate had been labeled
    in the No. 2 carbon as indicated by the asterisk,
    the ribose would be labeled in the No. 1 carbon
    and in no other carbon, as indicated. Such ex-
    periments have been carried out, for example
    by Marks and Feigelson,[28] in the liver of intact
    rats, and the following relative values for C^{14}
    were found in ribose of RNA following the in-
    jection of glucose-2-C^{14}:

Table 5.1   PENTOSE LABELLING FROM GLUCOSE-2-C^{14}

Carbon No.	1	2	3	4	5
Relative C^{14} (calc. for oxidative path.)	100	0	0	0	0
Relative C^{14} (observed)	23	76	3	0	0

The data show that the oxidative pathway alone
is inadequate to account for the labeling pattern
observed.

2. Non-oxidative Pathway. On the basis of the
    reactions shown in Figs. 5.2-5.4 there does
    not seem to be any balanced reaction scheme
    by which 1, 2, or more moles of fructose-6-
    phosphate can be converted to ribose-5-phos-
    phate non-oxidatively without involving the
    Embden-Meyerhof enzymes. The simplest in-
    volvement appears to require phosphofructo-
    kinase and one mole of ATP, or catalytic
    amounts of ATP and DPN. Omitting DPN, the
    reaction sequence can be written as follows:

a. Fructose-6-phosphate ⇌ Erythrose-4-phosphate + C_2
b. Fructose-6-phosphate ⇌ Glyceraldehyde-3-phosphate + C_3
c. Erythrose-4-phosphate + C_3 ⇌ Sedoheptulose-7-phosphate
d. Glyceraldehyde-3-phosphate + C_2 ⇌ Xylulose-5-phosphate
e. Xylulose-5-phosphate ⇌ Ribulose-5-phosphate
f. Ribulose-5-phosphate ⇌ Ribose-5-phosphate
g. Sedoheptulose-7-phosphate ⇌ Ribose-5-phosphate + C_2
h. Fructose-6-phosphate + ATP → ADP + Fructose-1:6-diphosphate
i. Fructose-1:6-diphosphate ⇌ Glyceraldehyde-3-phosphate +
                                    Dihydroxyacetone phosphate
repeat (d) Glyceraldehyde-3-phosphate + C_2 ⇌ Xylulose-5-phosphate
repeat (e) Xylulose-5-phosphate ⇌ Ribulose-5-phosphate
repeat (f) Ribulose-5-phosphate ⇌ Ribose-5-phosphate

Add: 3 Fructose-6-phosphate + ATP → ADP +
      Dihydroxyacetone phosphate + 3 Ribose-5-phosphate

In the presence of DPN and inorganic phosphate the system would regenerate ATP from ADP and would convert the dihydroxyacetone phosphate to lactic acid via glyceraldehyde-3-phosphate presumably without affecting the stiochiometric conversion of fructose-6-phosphate to ribose-5-phosphate in the reactions written above. However, the presence of triosephosphate isomerase (Fig. 5.5) will tend to convert glucose carbons 1, 2, and 3 to carbons 6, 5, and 4 and vice versa, which accounts for some of the unexplained C^{14} distributions described below. If the fate of a C^{14} carbon in position 2 of the fructose molecules (shown by an asterisk) or C^{14} from labelled CO_2 (shown by a small o) or from position 6 (shown by a small x) is followed into ribose-5-phosphate the following "simplest" scheme can be worked out with all carbons accounted for, corresponding to the equations given above:
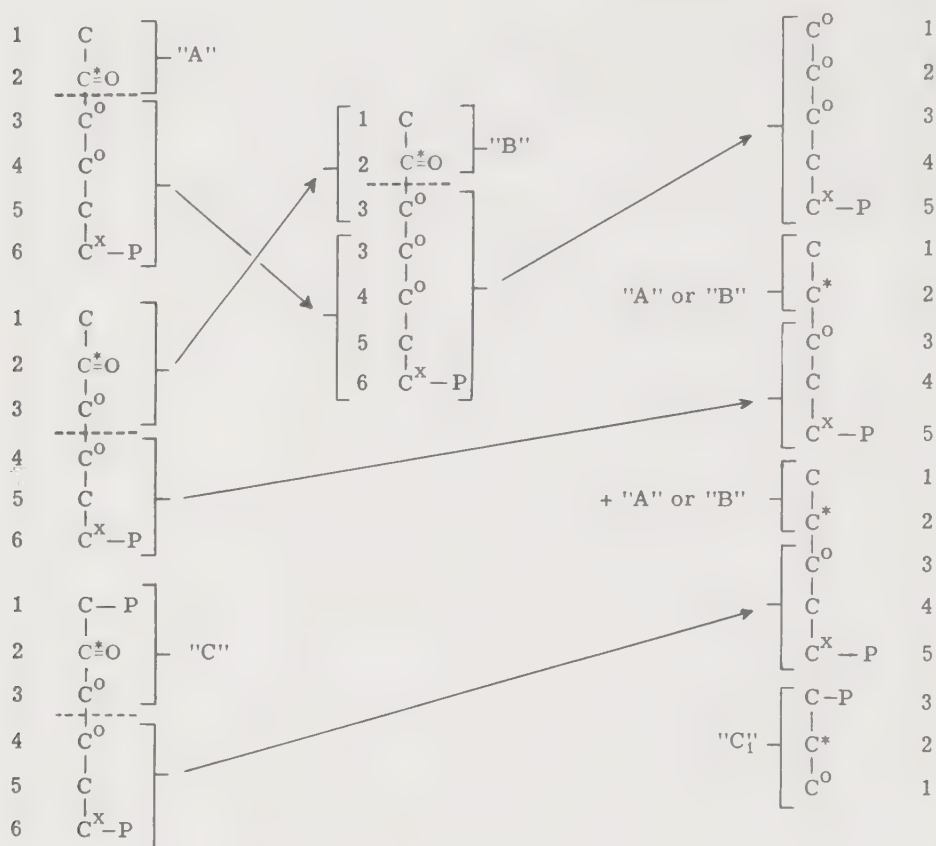
Fig. 5.6 Tracing the labeled carbon atoms in the non-oxidative pathway of ribose formation. This mechanism provides the basis for calculating the relative labeling shown in Tables 5.2 and 5.3 cf. [18] Three molecules of fructose-6-phosphate are converted to three molecules of ribose-5-phosphate and one molecule of triose phosphate. C^{14} originating in fructose in positions 2, 3 and 4, and 6 is symbolized here by *, °, and ×, respectively.

According to the above scheme, carbon 2 of fructose-6-phosphate will appear exclusively in carbon 2 of ribose-5-phosphate and in no other carbon. (Depending on lack of utilization of dihydroxyacetone phosphate in these reactions. If this utilization occurred, carbon 4 of ribose-5-phosphate would be labeled, but the data in Table 5.1 show that there was no labeling in carbon 4). It is now possible to compare

the observed data of Fig. 5.1 with the data cal-
culated from Fig. 5.6, the non-oxidative path-
way, and for a combination of the oxidative and
non-oxidative pathways.

Table 5.2. PENTOSE LABELLING FROM GLUCOSE-2-C^{14}

Carbon No.	1	2	3	4	5
Relative C^{14}, observed (Table 5.1)	23	76	3	0	0
Relative C^{14}, calc. oxidative (Table 5.1)	100	0	0	0	0
Relative C^{14}, calc. non-oxidative (Fig. 5.6)	0	100	0	0	0
Relative C^{14}, calc. for oxidative / non-oxidative = 1:1	50	50	0	0	0
" " " = 1:2	33	67	0	0	0
" " " = 1:3	25	75	0	0	0
" " " = 1:4	20	80	0	0	0
" " " = 1:5	17	83	0	0	0

From the above table, it is clear that by jug-
gling the proportion of oxidative to non-oxida-
tive pathways, the calculated ratios could
always be made to match the observed data.
The main conclusion to be drawn is that both
pathways must be operative to account for the
observations, and secondly that there is rela-
tively little mixing of the radioactivity into car-
bons 3, 4, and 5 of the pentose. In the work
referred to[28] the glycogen was also isolated
after giving glucose-2-C^{14} and the glucose was
degraded to give relative values in carbons 1-6
of 14, 66, 5, 6, 7, 5. These data suggest that
much of the glucose of glucose-2-C^{14} moves
into the pentose pool with label in carbons 1:2
as 25:75 and that these carbons then move back
to glucose with label in carbons 1 and 2, with
the additional label in carbon 2 coming from
glucose-2-C^{14} that moved directly into glycogen.

Many studies have been carried out with label-
ed bicarbonate as the precursor of glucose and
pentose. It has been widely confirmed that
labeled CO_2 moves into C-1 of triose and is
therefore equally distributed between C-3 and
C-4 of glucose obtained from glycogen. It is
of interest to note the pentose labelling in RNA
from rat liver following administration of
$C^{14}O_2$ as carried out by both Marks and Feigel-
son[28] and Bernstein[4]. In both instances, glyco-
gen was shown to be labeled in both C-3 and
C-4 in nearly equal proportions with essentially
no label in the other carbons.

Table 5.3.   PENTOSE LABELLING FROM $C^{14}O_2$

Carbon No.	1	2	3	4	5
Relative C^{14}, observed[28]	40	73	100	9	2
Relative C^{14}, observed[4]	22	49	100	6	2
Relative C^{14}, calc. oxidative	0	100	100	0	0
Relative C^{14}, calc. non-oxidative	33	33	100	0	0
Relative C^{14}, calc. for oxidative non-oxidative = 1:3	25	50	100	0	0

The assumption of 1 part oxidative pathway to
3 parts non-oxidative pathway in rat liver was
based upon the glucose-2-C^{14} data in Table 5.1.
The available data suggest that glucose-2-C^{14}
is the preferable precursor in such studies,
since the calculated results for the oxidative
versus the non-oxidative pathways show clean
cut differences (Table 5.2) not seen in the case
of the glucose labeled with CO_2.

Similar studies with acetate-1-C^{14} and glucose-
6-C^{14} in E. coli gave results that also suggest
about 3 parts non-oxidative to 1 part oxidative
synthesis of ribose.[3]

GLUCOSE   GLUCOSE-1-P ⇌ GLYCOGEN

ATP   TPN   TPN

GLUCOSE-6-P → 6-P-GLUCONIC → RIBULOSE-5-P + CO_2

⇌ FRUCTOSE-6-P   XYLULOSE-5-P   RIBOSE-1-P ⇌

ATP   TRIOSE-P

FRUCTOSE-1:6-DP   RIBOSE-5-P

TRIOSE-P + ∼ C_3 ERYTHROSE-4-P + ∼ C_2 ATP

ADP   SEDOHEPTULOSE-7-P

PYRUVIC ACID

CO_2 LACTIC ACID   PHOSPHORIBOSYL PYROPHOSPHATE

ALTERNATIVE PATHWAYS   + BASES

WHERE? WHEN? WHY?   NUCLEOTIDES + PP

Fig. 5.7. The alternative pathways between glucose and the ribose in nucleotides. The details of the Embden-Meyerhof glycolytic pathway below fructose-1,6-diphosphate, and the details of the Krebs citric acid cycle between pyruvate and CO_2 are not shown, but the over-all conversions are indicated by dashed lines. Triose phosphate (glyceraldehyde 3-phosphate) is shown in two places on the chart to avoid crossing lines. Reversible arrows at the periphery indicate connections to compounds not shown on the chart.

A chart summarizing the oxidative and non-oxidative pathways is shown in Fig. 5.7. It should be emphasized that the wide variations in the estimates of the relative importance of the HMP shunt and the non-oxidative pathway may be related to factors affecting the reoxidation of TPNH generated in the HMP shunt, as emphasized by De Duve and Hers[8] and Utter[47]. The many papers in this field will not be summarized here as it is our purpose to emphasize the coexistence of the two pathways and not their relative values in various tissues.

E.   Formation from Ribose-1-phosphate and Ribose

The preceding sections were concerned with the de novo synthesis of ribose-5-phosphate. This compound can also be formed from ribose-1-phosphate by the action of a phosphoribomutase[16] or from free ribose by a ribokinase[1], an enzyme requiring Mg ions and ATP. This enzyme was found in highest amounts in liver, with much lower levels in spleen, kidney and brain and none in heart muscle.

IV.   5-PHOSPHORYL-α-D-RIBOFURANOSE-1-PYROPHOS-PHATE (PRPP)

This compound was first isolated by Kornberg, Lieberman and Simms[23] and has been shown to be of widespread significance in the biosynthesis of the purine and pyrimidine nucleotides, both at the level of the de novo synthesis of each class of compounds and also at the level of the utilization of the preformed bases. The compound is formed by the following reaction:
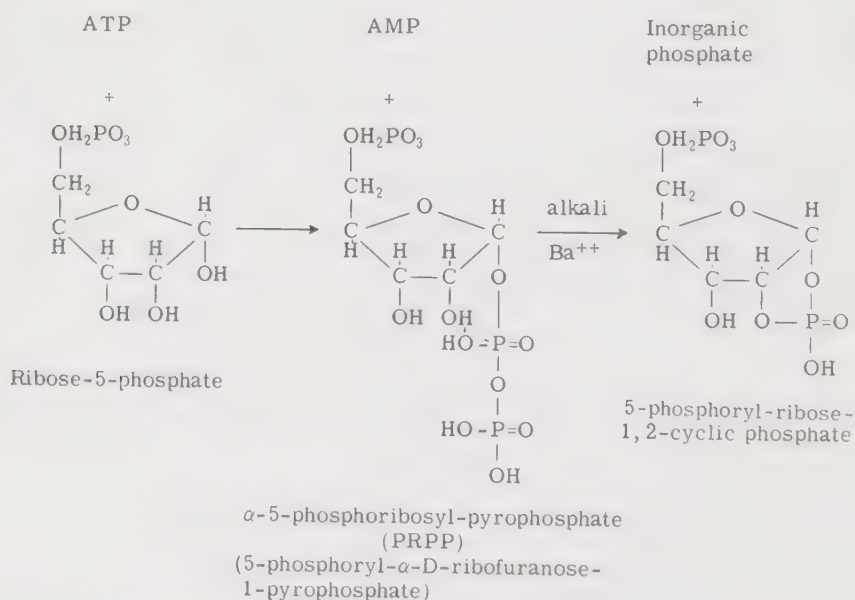

α-5-phosphoribosyl-pyrophosphate
(PRPP)
(5-phosphoryl-α-D-ribofuranose-
1-pyrophosphate)

Fig. 5.8. Enzymatic formation[23] of PRPP and its chemical degradation[22]. The compound also breaks down at neutral pH at 100° in a few minutes. [39]

In this reaction it will be noted that a pyrophosphate group is transferred to carbon 1 of the ribose-5-phosphate. It was shown by Remy, Remy and Buchanan[39] that the pyrophosphate is attached in α linkage on the basis of the fact that in alkali one phosphate is split out with the formation of a cyclic monophosphate between Carbons 1 and 2 of the ribose. Further confirmation has been provided by Khorana, Fernandes and Kornberg[22], and the compound has been chemically synthesized by Tener and Khorana[46].

In the utilization of PRPP the nitrogen of the base that displaces pyrophosphate ends up in the β configuration to form a 5' nucleotide. The specific reactions utilizing PRPP will be described in the appropriate sections of the next chapter.

V. RIBOSE-1-PHOSPHATE

The formation of ribose-1-phosphate can occur in two ways. It can be formed from ribose-5-phosphate by the action of phosphoribomutase[16] an enzyme that so far has been found in smooth muscle and in blood. This pathway represents an alternative to PRPP formation but knowledge of the distribution of the respective enzymes is still rudimentary. The second pathway of ribose-1-phosphate formation is by means of a reaction first studied by Kalckar[21] in 1947. The enzyme purine nucleoside phosphorylase[21] catalyzes the following reaction, which is reversible and favors the utilization rather than the formation of ribose-1-phosphate:

purine ribonucleoside + inorganic phosphate = purine + ribose-1-phosphate.

A similar enzyme that acts on uracil but not on cytosine has been described for bacteria[31] and for rat liver[9].

Since both the purine and the pyrimidine nucleoside phosphorylases catalyze reactions that involve very little change in free energy, it is clear that the direction of the reactions will be strongly affected by the fate of the products on either side of the reaction. In mammalian

organisms the liver very actively degrades both free
purines and pyrimidines and it seems rather likely that
the overall result will be to further the conversion of the
nucleosides to ribose-1-phosphate and free base. How-
ever in micro-organisms and other forms of life that do
not actively degrade the free bases the formation of
ribose-1-phosphate and its utilization for nucleoside
synthesis may constitute a significant biosynthetic path-
way, in competition with the pathway that utilizes PRPP.

VI.   2-DEOXYRIBOSE-1-PHOSPHATE

In 1951 Racker[34] reported the presence of an aldolase in
E. coli and in animal tissues. The enzyme catalyzes a
condensation between glyceraldehyde-3-phosphate and
acetaldehyde to give 2-deoxyribose-5-phosphate. More
recently, Boxer and Shonk[6] report the occurrence of the
enzyme in rat liver and in hepatoma and suggest that
threonine may be the source of the acetaldehyde moiety
by an aldolase cleavage that also yields glycine.

A phosphodeoxyribomutase[12, 27] converts deoxyribose-5-
phosphate into deoxyribose-1-phosphate in both animal
and bacterial systems.

The formation and utilization of deoxyribose-1-phosphate
can also occur through the action of purine deoxyribonu-
cleoside phosphorylase in liver[11, 12] and in thymus and
E. coli[27]:

purine + deoxyribose-1-phosphate = purine deoxyriboside + inorganic phosphate.

Similar enzymes react with the pyrimidines uracil and
thymine but as in the case of the ribosides, the cytosine
derivatives do not participate.[9]

The significance of Racker's enzyme and the possibility
of forming deoxyribonucleosides through the coordinated
action of the mutase and phosphorylases seems diminish-
ed by evidence that there is a direct reduction of the ribo-
side moiety to deoxyribose while certain bases remain

attached as described in the next section. The occurrence of enzymes that transfer deoxyribose from one base to another has been described under the name trans-N-deoxyribosylase by Roush and Betz[42] who confirmed and extended the work of MacNutt. At present there is insufficient evidence to decide whether the Racker enzyme and the trans-N-deoxyribosylase play significant roles in the synthesis of the deoxyribonucleotides, but in all probability they represent alternative pathways in at least some types of cells.

VII. 5'-DEOXYRIBOTIDES

Three types of study suggest that the deoxyribonucleotides may be formed from ribotides rather than through the intermediate formation of deoxyribosephosphates. In several studies involving labeled glucose it was found that the various carbons in the deoxyribose of DNA had the same relative radioactivities as the corresponding carbons in the ribose of RNA[2, 3, 5]. These studies could best be explained if it were assumed that a fairly proximal precursor of DNA were formed from a proximal precursor of RNA, since it seemed most unlikely that intact RNA molecules could be transformed directly to DNA molecules.

The second line of evidence is fairly direct in that certain ribosides or ribotides having C^{14} in both the nitrogenous base and in the sugar moiety were found to appear in DNA without alteration of the ratio of the C^{14} in the base to the C^{14} in the sugar moiety. Such experiments were first carried out with totally labeled cytidine-C^{14} in the rat[41] and have subsequently been carried out with cytidylic and uridylic acids[40] and with uridine[37].

Finally, attempts have been made to determine by enzyme studies whether the reductive step is at the level of the nucleosides or at some higher level of phosphorylation. Although reductions have been reported with both ribosides and ribotides[10, 14, 15] Reichard[38] has presented data in favor of reduction of both uridine and cytosine at the ribotide level.

Since deoxy-UMP can be formed from deoxy-CMP by deamination[26] it might be thought that reduction of cytidine would suffice for the formation of both deoxy compounds. However, since many tissues lack deoxy-CMP deaminase it is likely that they form deoxy-UMP from UMP[33], as previously suggested by Reichard[38]. Further details on the interconversions among the nucleosides and nucleotides will be given in the next chapter but in discussing the biosynthesis of deoxyribose it is necessary to mention the evidence in favor of reduction at the ribotide level. While the evidence for direct reduction of the purine ribotides is meager, it would be necessary to have such reductions or to have transdeoxyribosidation between purines and pyrimidines to account for identical labeling in the sugar moieties of RNA and DNA[2, 3, 5].

VIII.   FUTURE STUDIES

Biochemists have provided multiple alternative pathways for the synthesis of nearly every important component of the nucleic acids, and the sugar moieties are outstanding examples of this generalization. Our knowledge now needs to be translated from what reactions can happen to terms that show what reactions do happen in particular organisms or tissues in the body. In establishing the latter, it will be necessary to use several different techniques, such as the use of isotopic tracers in intact cells or animals, plus the determination of each individual enzyme that can act on the intermediates involved.

REFERENCES

1. AGRANOFF and BRADY. J. Biol. Chem. 219:221 (1956).
2. BAGATELL, WRIGHT and SABLE. Biochim. Biophys. Acta 28:216 (1958).
3. BAGATELL, WRIGHT and SABLE. J. Biol. Chem. 234:1369 (1959).
4. BERNSTEIN. Biochim. Biophys. Acta 19:179 (1956).
5. BERNSTEIN and SWEET. J. Biol. Chem. 233:1194 (1958).

6. BOXER and SHONK. J. Biol. Chem. 233:535 (1958).
7. CIBA SYMPOSIUM, The Regulation of Cell Metabolism. London, 1959.
8. DeDUVE and HERS. Ann. Rev. Biochem. 26:149 (1957).
9. DeVERDIER and POTTER. J. Nat. Cancer Inst. 24:13 (1960).
10. EDMONDS. Fed. Proc. 17:215 (1958).
11. FRIEDKIN. J. Biol. Chem. 184:449 (1950).
12. FRIEDKIN and KALCKAR. J. Biol. Chem. 184:437 (1950).
13. GLOCK in Chargaff and Davidson. 2:247 (1954).
14. GROSSMAN. Fed. Proc. 17:235 (1958).
15. GROSSMAN and HAWKINS. Biochim. Biophys. Acta. 26:657 (1957).
16. GUARINO and SABLE. J. Biol. Chem. 215:515 (1955).
17. HAMMARSTEN. Z. physiol. Chem. 19:19 (1894).
18. HORECKER and MEHLER. Ann. Rev. Biochem. 24:207 (1955).
19. HORECKER et al. J. Biol. Chem. 218:745, 753, 769, 785, 795 (1956).
20. HORECKER and SMYRNIOTIS. J. Am. Chem. Soc. 74:2123 (1952).
21. KALCKAR. J. Biol. Chem. 167:477 (1947).
22. KHORANA, FERNANDES and KORNBERG. J. Biol. Chem. 230:941 (1958).
23. KORNBERG, LIEBERMAN and SIMMS. J. Biol. Chem. 215:389 (1955).
24. LEVENE and JACOBS. Ber. 42:1198 (1909) and Ber. 44:746 (1911).
25. LEVENE, MIKESKA and MORI. J. Biol. Chem. 85:785 (1930).
26. MALEY and MALEY. J. Biol. Chem. 234:2975 (1959).
27. MANSON and LAMPEN. J. Biol. Chem. 191:95 (1951); 193:539 (1951).
28. MARKS and FEIGELSON. J. Biol. Chem. 226:1001 (1957).
29. NIGAM, SIE and FISHMAN. J. Biol. Chem. 234:1955 (1959).
30. OVEREND and STACEY in Chargaff and Davidson 1:9 (1954).

31. PAEGE and SCHLENK. Arch. Biochem. Biophys. 40:42 (1952).

32. POTTER in Nord and Werkman. Advances in Enzymol. 4:201 (1944).

33. POTTER, PITOT and BRUMM. Fed. Proc. 19: (1960).

34. RACKER. Nature 167:408 (1951) and J. Biol. Chem. 196:347 (1952).

35. RACKER. Adv. Enzymol. 15:141 (1954).

36. RACKER, de la HABA, and LEDER. Arch Biochem. Biophys. 48:238 (1954).

37. REICHARD. Acta Chem. Scand. 11:11 (1957).

38. REICHARD. Biochim. Biophys. Acta. 27:434 (1958).

39. REMY, REMY and BUCHANAN. J. Biol. Chem. 217:885 (1955).

40. ROLL, WEINFELD and CARROLL. J. Biol. Chem. 220:455 (1956).

41. ROSE and SCHWEIGERT. J. Biol. Chem. 202:635 (1953).

42. ROUSH and BETZ. J. Biol. Chem. 233:261 (1958).

43. SEEGMILLER and HORECKER. J. Biol. Chem. 194:261 (1952).

44. SMITH and DUNN. Biochim. Biophys. Acta 31:573 (1959).

45. TABACHNICK, SRERE, COOPER and RACKER. Arch. Biochem. Biophys. 74:315 (1958).

46. TENER and KHORANA. J. Am. Chem. Soc. 80:1999 (1958).

47. UTTER. Ann Rev. Biochem. 27:245 (1948).

Chapter VI
The Biosynthesis of the
Purines and Pyrimidines

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Purines and Pyrimidines

I.   INTRODUCTION

It has been known for many years that the purines and
pyrimidines are not <u>essential</u> <u>nutrients</u> for the vast
majority of living cells, yet it is equally clear that
they are <u>essential</u> <u>metabolites</u> for all forms of life.
The distinction between essential nutrients and essen-
tial metabolites was admirably discussed by Knight in
1945[46] and quoted by Potter and Heidelberger in 1950
as follows:[69] "....The substances which an organism
takes from its nutrients are used as material for build-
ing up the new cells. These cells carry out a complex
interwoven series of processes, which is the life of
those cells, and consists in taking compounds from the
environment and synthesizing other compounds to make
new cells. The extent and rate of multiplication of
new cells will depend on the efficiency with which the
processes of construction are carried out. This ef-
ficiency (here used in the general and not only thermo-
dynamic sense) will clearly depend partly on the avail-
ability of the materials of construction. This will in
turn depend upon the rates of utilization and synthesis
of the various materials of the enzyme systems whose
continued functioning is the life of the cells. The fun-
damental biochemical processes of cell life - the es-
sential metabolism of the cells - form the cardinal
feature, and certain of these processes are common
to the widest variety of cell. Where organisms may
differ, however, is in the means whereby the materials
for these processes are acquired. But here a sharp

metaphysical distinction into 'acquired from the environment' or 'synthesized by the cell' is not possible. For it is clear that a certain rate of synthesis might be too slow to yield a required rate. Effectively then the cell would depend upon an external source of supply, to a degree which would be relative to the rate of synthesis of this substance. Hence a given substance, required as a component of one of the essential metabolic processes, might appear in three different roles as a component of the nutrients. It might appear: (1) as an 'essential' nutrient, when its rate of synthesis by the cell was so slow as to be insignificant; (2) as a growth stimulant, when its rate of synthesis was somewhat faster but still slow enough to be a limiting factor; or (3) as a substance not required at all for nutrition, because the cell could synthesize it so fast that it was not a limiting factor in growth. It is the metabolic process which is the essential thing and the compounds used in carrying it out are essential metabolites, i.e. the substrates used for the process, or the substance which forms parts (prosthetic groups, etc.) of the enzyme systems which carry out these essential reactions...".

The essential metabolite concept is nowhere more significantly and abundantly documented than in the case of the purines and pyrimidines. The point to be emphasized is that an essential metabolite for a given form of life is an absolutely necessary constituent of its protoplasm; it is needed for the synthesis of compounds without which the organism could not exist. In many organisms the essential metabolite can be synthesized from simpler building blocks, and in these organisms it is not an essential nutrient because it does not need to be present in the food supply or in the medium; however some organisms have undergone one or more mutations that have left them unequipped to carry out the synthesis of certain essential metabolites. These organisms can live if the compound in question is present in the medium; for them the compound is an essential nutrient.

The foregoing discussion might leave the impression
that a given organism which can synthesize a purine,
for example adenine, from smaller building blocks
might lack the enzymatic equipment to utilize adenine
that might be present in the medium. However experi-
ments have shown that micro-organisms and probably
cells in general utilize adenine even if they are able to
synthesize it, and in fact they have built-in feedback
controls (Chapter XII) that permit them to shut off
their synthetic pathways for any essential metabolite
that is offered in the medium. It is customary to refer
to the synthesis of essential metabolites from the
smallest building blocks (glycine, formate, CO_2, NH_3)
as de novo synthesis, to distinguish it from utilization
of preformed building blocks (e.g. adenine, uracil).
The utilization of preformed building blocks has been
referred to as the "salvage" pathway by Kornberg[47]
who was inclined to minimize its importance. However,
it now seems more appropriate to conclude that these
pathways will be used by nearly all cells whenever the
bases are available, while the de novo pathways will
be used only when the bases are not available (see
Chapter XII). In general the term "preformed" rather
than "salvage" pathway is found in the literature.
Higher organisms have both de novo and "preformed"
pathways for both purine and pyrimidine biosynthesis
present in varying ratios in different tissues and in
addition have extremely vigorous enzymes for breaking
down the purines and pyrimidines, chiefly in the liver
(Chapter VIII). Many details of the de novo and the pre-
formed pathways have been worked out for purines and
pyrimidines in recent years and it appears that except
for minor details the pathways are nearly identical in
microorganisms and higher animals. While less data
are available for plants it is likely that both pathways
can occur. Information on the feedback controls will
be described in Chapter XII.

The biosynthesis of the purines and pyrimidines was
discussed by Reichard in 1954[72] when the main outlines
of the pathways were just taking shape. More recently

Reichard has reviewed purine biosynthesis[76] and Buchanan and Hartman[12] have reviewed purine biosynthesis in the same volume with a combined total of 336 references. An excellent series of original research reports on de novo synthesis of purines has been published in the J. Biol. Chem. under the general heading Biosynthesis of the Purines I-XXVI during the period 1946-1959. These references are given in Table 6.1 in chronological order.[10] The references listed thus far have been mainly concerned with de novo syntheses.

Table 6.1. BIOSYNTHESIS OF THE PURINES

Papers I-XXVI and 4 preceding papers in the Journal of Biological Chemistry by J. M. Buchanan and colleagues, 1946-1959.

Paper No.	Subject	Vol. Page	Year	Paper No.	Subject	Vol. Page	Year
a*	Uric acid	166: 395	(1946)	XII	AIR	224:1005	(1957)
b*	" "	166: 781	(1946)	XIII	FGAM	224:1019	(1957)
c*	" "	173: 69	(1948)	XIV	FGAM	225: 157	(1957)
d*	" "	173: 81	(1948)	XV	FGAM	225: 163	(1957)
I	Hx	196: 499	(1952)	XVI	AICAR	228: 201	(1957)
II	AICA	196: 513	(1952)	XVII	IMP	228: 215	(1957)
III	IMP	202: 241	(1953)	XVIII	IMP	229: 603	(1957)
IV	IMP	202: 253	(1953)	XIX	FGAR	229: 613	(1957)
V	IMP	203: 583	(1953)	XX	FAICAR	229: 627	(1957)
VI	R-1-P	217: 183	(1955)	XXI	PRA	233: 451	(1958)
VII	PRPP	217: 875	(1955)	XXII	GAR	233: 456	(1958)
VIII	PRPP	217: 885	(1955)	XXIII	SAICAR	234:1791	(1959)
IX	Purine-N	220: 369	(1956)	XXIV	AIR	234:1799	(1959)
X	Purine-N	220: 379	(1956)	XXV	AICAR	234:1806	(1959)
XI	FGAR	221:1057	(1956)	XXVI	C$_1$	234:1812	(1959)

* These 4 papers were not numbered in the original publications. The bibliographies of the 30 papers in this table provide references to contemporary papers by Greenberg and others, many of which are omitted from the text of this chapter.

The utilization[9] of preformed purines, pyrimidines and their corresponding ribonucleosides and deoxyribonucleosides brings in a number of alternative pathways that involve the reaction of the free bases (except cytosine) with ribose-1-phosphate, deoxyribose-1-phosphate, or phosphoribosylpyrophosphate. The

various alternative pathways appear to be widely dis-
tributed in nature and their relative importance in any
one cell or animal tissue does not appear to have been
worked out, but in any case will be strongly dependent
upon the conditions of the experiment.

II.   HISTORICAL BACKGROUND FOR PURINE BIOSYN-
THESIS [11], [12], [72], [76]

Aside from the fact that animals were known to be able
to grow on diets containing no pyrimidines or purines, [9]
little was known about the synthesis of these compounds
until the use of isotopic tracers became widespread
shortly after World War II.

The pre-isotope era included some important studies
that set the stage for later advances by Buchanan and
by Greenberg and their coworkers, who used isotopic
tracers extensively. It was known that the end product
of purine metabolism in birds is uric acid, and the
early studies centered on this compound, using tissues
from pigeons. It was shown that pigeon liver slices
produced a substance that could be converted to uric
acid by kidney slices[84]. H. A. Krebs had just formu-
lated the "ornithine cycle" as a mechanism for urea
production in animals[52] and was now interested in the
mechanism of uric acid production in birds. It was
shown that the substance accumulating in pigeon liver
slices was hypoxanthine, which was not further metab-
olized because of the absence of xanthine oxidase,
which was shown to be present in kidney slices. [17] The
yield of hypoxanthine was shown to be increased by
lactate and pyruvate[17] and by oxalacetate and glutamine[66],
a combination that would generate aspartic and glutamic
acids. All of these findings played an important role in
later isotope studies which were initiated some 6 years
later.

Meanwhile important studies were carried out with
microorganisms. In 1945 it was shown that E. coli

grown in the presence of sulfonamides accumulated a diazotizable amine[86] that was identified as 4-amino-5-imidazole-carboxamide by Shive et al[85], who recognized the probable relation to purine biosynthesis since the compound is equivalent to hypoxanthine with carbon 2 missing. Many additional studies with microorganisms supported the idea that the compound was an intermediate in purine biosynthesis. After the isotopic studies got under way, it was shown that the true intermediate is not the free carboxamide but the 5' ribonucleotide as illustrated in Fig. 6.2 later in this chapter, and Greenberg[29] showed that in the sulfonamide-inhibited cultures of E. Coli the compound that accumulates is the ribonucleoside of the carboxamide. The reaction that is blocked is the formylation of the carboxamide ribotide; as soon as this compound begins to accumulate it is dephosphosphorylated to the riboside and excreted into the medium. The riboside is very labile and the earlier isolation procedures yielded the free carboxamide. In a mutant lacking the enzyme for the formylation step, the riboside is also excreted[25]. These studies with micro-organisms paralleled the isotopic experiments with pigeon liver and were very important to their progress.

The initial isotopic attack on the problem of purine synthesis was carried out by Buchanan and coworkers by administering labeled compounds to live pigeons and isolating uric acid from the excreta. It is of interest that the compounds given included not only lactate and pyruvate[17] but also acetate, CO_2, formate, and glycine. The latter compound is the simplest amino acid and was undoubtedly the only labeled amino acid available at the time. It must have been administered on the basis of availability rather than historical precedent. The same must have been true for CO_2 and formate, yet these 3 compounds proved to be the critical building blocks in the early stages of biosynthesis of the purine ring. This happy result is probably not a coincidence but is a reminder that biosynthetic pathways tend to occur by the addition of fairly simple groups of atoms at each

step. The experimental technique consisted in giving each of the labeled precursors one at a time and determining the exact localization of the isotope in specific atoms in the uric acid molecule. The latter depended upon methods of degrading uric acid in such a way that each atom could be identified.[7,8,10d]

On the basis of these preliminary studies with labeled precursors of uric acid it was shown that carbons 2 and 8 came from formate, carbon 6 came from CO_2, and carbon 4 came from carbon 1 of glycine[10a-d]. These studies were amply confirmed in other laboratories[72]. During this period it was also established that nitrogen 7 came from glycine. These early studies can be looked at in the light of the final picture of de novo synthesis as shown in Fig. 6.1.
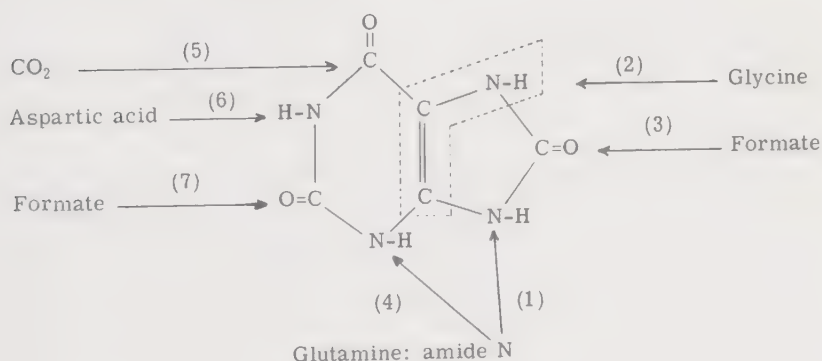
Fig. 6.1. De novo synthesis of the purine molecule including the earliest data obtained with isotopes entering the uric acid molecule (steps 2, 3, 5, and 7) and the most recent results. The numbers in parentheses show the order in which the individual atoms or groups enter the purine structure according to details given in Fig. 6.2.

At this point G. R. Greenberg[26,27], using pigeon liver homogenates, showed that radioactive formate and CO_2 were incorporated into the hypoxanthine (shown earlier to accumulate in slices[17]) and made the important discovery that the hypoxanthine was not a primary product but was derived from the ribonucleotide of hypoxan-

thine, i.e. inosinic acid. This compound was well
known as the deamination product of adenylic acid and
at this time there was no reason to believe that it was
a precursor of adenylic acid. It became the focus of
attention and has remained at the crossroads of purine
metabolism ever since, as will be shown. From these
early studies with homogenates, Greenberg and Buchan-
an and their respective coworkers moved on to soluble
enzyme systems, isolated the individual enzymes, and
isolated the intermediate products in the de novo synthe-
sis of the purines. During all this time the interaction
of the reactions involved in the utilization of preformed
purines tended to complicate the results[10V-VIII] but the
de novo pathway was separated from the preformed
pathway and worked out in great detail. It will be brief-
ly presented in the next section.

III.   THE BUCHANAN-GREENBERG PATHWAY FOR THE DE NOVO SYNTHESIS OF PURINE RIBONUCLEO-TIDES

The discussion of the details of the de novo pathway
involves the mention of a large number of intermedi-
ates that can be referred to most easily by the use of
their abbreviated names. In addition to the abbrevi-
ated forms, the trivial names are very useful, and
these must be referred to the chemical names that
describe the specific structures. Since the chemical
names are used in the titles of many of the papers in
Table 6.1 it is useful at this point to tabulate the three
designations for each compound in the pathway (Table
6.2). The structures can be obtained from Fig. 6.2.
A simplified version of the synthetic pathway is given
in Fig. 6.3 in which equilibrium reactions are shown
by horizontal arrows, and all energy-requiring steps
are shown by vertical or diagonal arrows in the main
pathway. The simplified scheme shown in Fig. 6.3 is
intended to give a better perspective of the overall de
novo pathway of purine biosynthesis and to integrate
this scheme with the preformed pathways, which will
be discussed in the next section.

Table 6.2.   DESIGNATIONS OF INTERMEDIATES IN THE DE NOVO PATHWAY
OF PURINE BIOSYNTHESIS, LISTED IN THE ORDER OF THEIR
FORMATION

Abbrevia-tions	Trivial Names	Chemical Names
PRPP	5-phosphoribosylpyrophosphate	5-phosphoryl-α-D-ribofuranose 1-pyrophosphate
PRA	5-phosphoribosylamine	
GAR	glycinamide ribotide	2-amino-N-ribosylacetamide 5'-phosphate
FGAR	formylglycinamide ribotide	2-formamido-N-ribosylacetamide 5'-phosphate
FGAM	formylglycinamidine ribotide	2-formamido-N-ribosylacetamidine 5'-phosphate
AIR	5-aminoimidazole ribotide	5-amino-1-ribosylimidazole 5'-phosphate
Carboxy-AIR	5-amino-4-imidazolecarboxylic acid ribotide	5-amino-1-ribosyl-4-imidazole-carboxylic acid 5'-phosphate
SAICAR	5-amino-4-imidazole-N-succino-carboxamide ribotide	N-(5-amino-1-ribosyl-4-imidazole-carbonyl)-L-aspartic acid 5'-phosphate
AICAR	5-amino-4-imidazole-carboxamide ribotide	5-amino-1-ribosyl-4-imidazole-carboxamide 5'-phosphate
FAICAR	5-formamido-4-imidazole-carboxamide ribotide	5-formamido-1-ribosyl-4-imidazole-carboxamide 5'-phosphate
IMP	inosinic acid	inosine 5'-phosphate
SAMP*	adenylosuccinic acid	
AMP	adenylic acid	adenosine 5'-phosphate
XMP*	xanthylic acid	xanthosine 5'-phosphate
GMP	guanylic acid	guanosine 5'-phosphate

* SAMP and XMP lie on alternative pathways between IMP and AMP and GMP, respectively, as shown in Fig. 6.2.
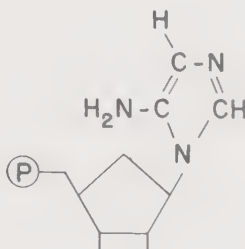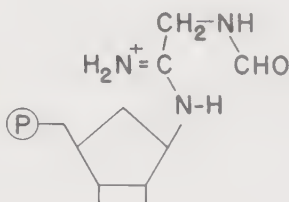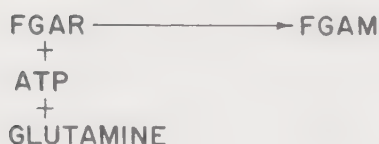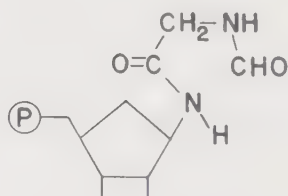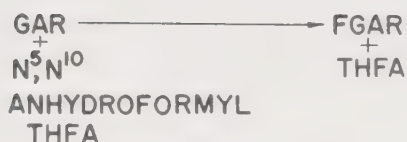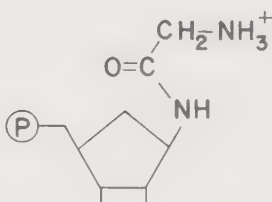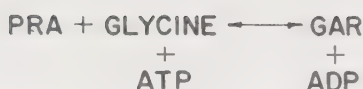
Fig. 6.2a. Buchanan-Greenberg pathway for the biosynthesis of the purine ribonucleo-
tides. (Initial stages). Ribose is shown as a pentagon and phosphate is shown
as the letter P within a circle. The meanings of the abbreviations are given
in Table 6.2. THFA = tetrahydrofolic acid.
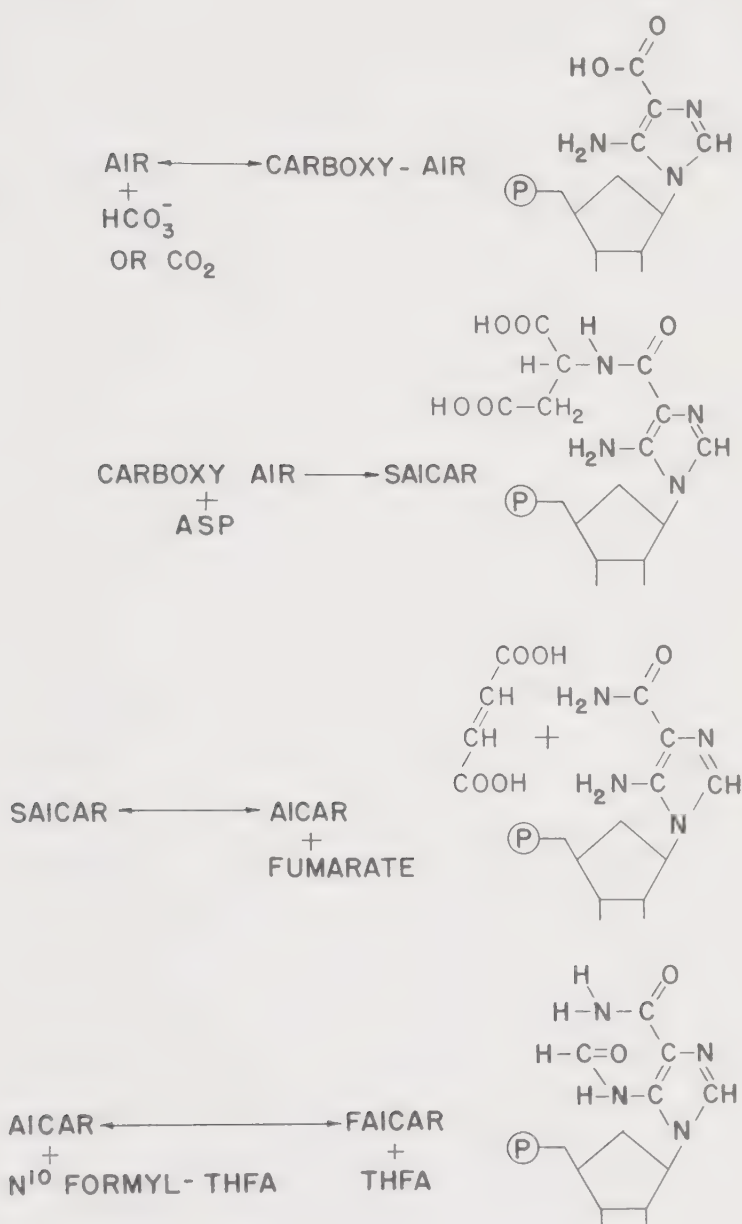
Fig. 6.2b. Buchanan-Greenberg pathway for the biosynthesis of the purine ribonucleotides. (Intermediate stages). Abbreviations as in Fig. 6.2a.
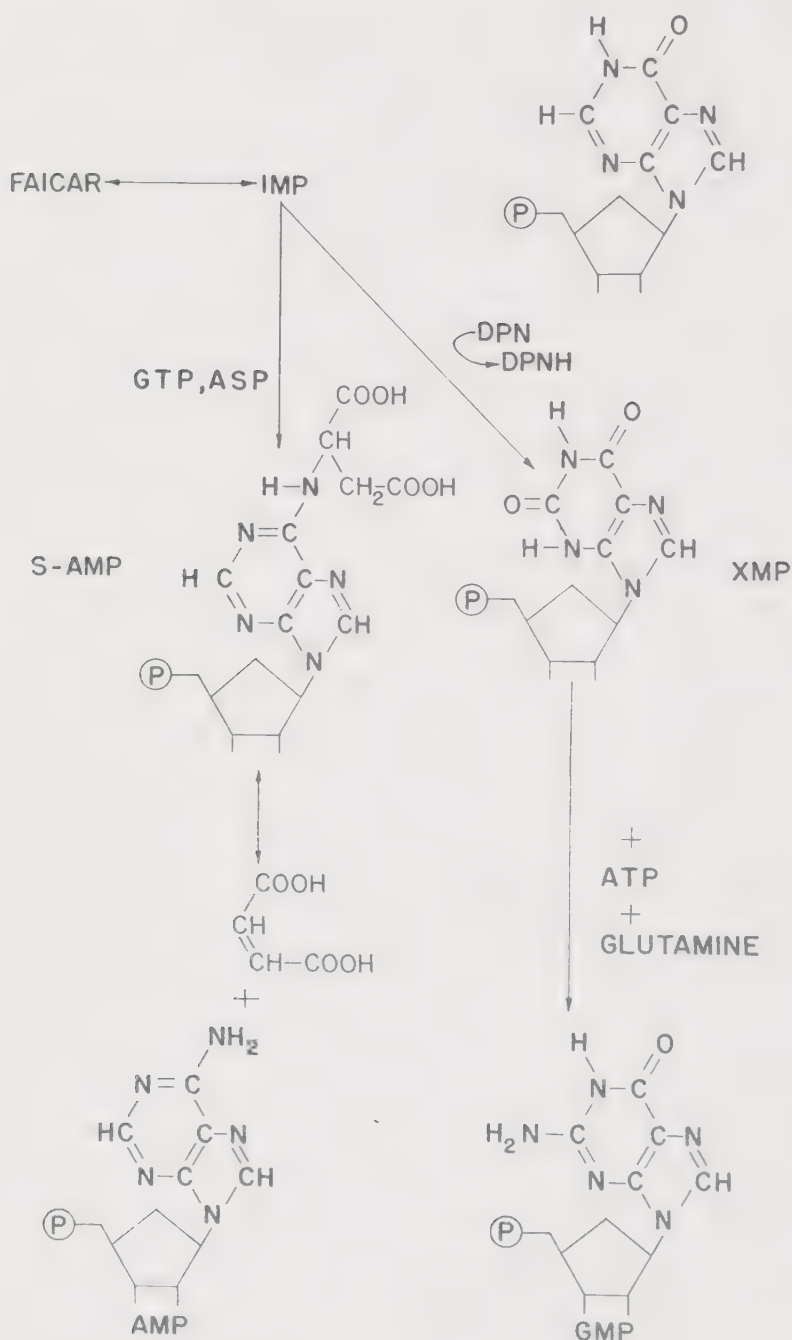
Fig. 6.2c. Buchanan-Greenberg pathway for the biosynthesis of the purine ribonucleotides. (Final stages). Abbreviations as in Fig. 6.2a.
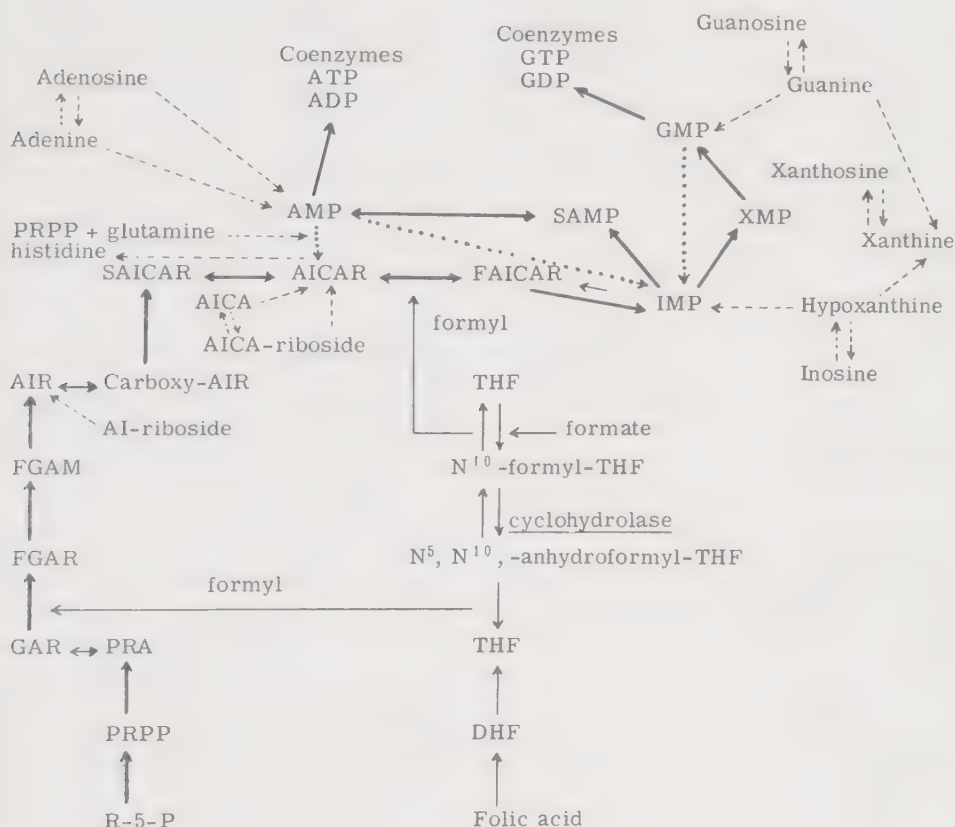
Fig. 6.3. Simplified Buchanan-Greenberg reaction sequence for the de novo synthesis of the purine ribonucleotides, with preformed pathways added as dashed lines, with terminal cycles in dotted lines, and with most of the coenzymes and co-substrates omitted. The solid horizontal arrows in the main pathway indicate equilibrium mixtures, while the reversibility of the reactions shown by solid vertical arrows in the main pathway has not been demonstrated. Compare with Fig. 6.2. Catabolic conversion of adenine to hypoxanthine and xanthine to uric acid not shown. Pathways leading to deoxyribonucleotides are also omitted.

One of the important points to note is that the goal of the synthetic pathways is the production of adenylic acid and guanylic acid, the two major purine ribonucleoside 5'-monophosphates. From these two compounds all of the higher phosphates and coenzymes are derived. The details shown in Fig. 6.3 represent a small portion of Fig. 2.9, shown in Chapter II, in the arrows numbered 1, 2, 12 and 16.

A second important point to note is the strategic position of inosinic acid, IMP. A single de novo pathway leads to IMP, and from this compound only minor changes are needed to yield either AMP or GMP. Apparently the monophosphates are the compounds formed by the biosynthetic pathways, none of which appear to lead to the higher phosphates except by way of the monophosphates. The ribose-5-phosphate moiety is the basic structure around which the intermediates in the synthesis of the purine ribonucleotides are formed. All of the intermediates between PRA and FAICAR have been reported in the form of the 5'-monophosphates and no reports of higher phosphates of these compounds have appeared.

The reciprocal coenzyme functions of ATP in the synthesis of GMP and GTP in the synthesis of AMP were included in Fig. 6.2[53].

The terminal cycles, by which GMP and AMP can both be converted back to IMP by different routes from the synthetic pathways provides a neat method for interconverting the two bases. The conversion of AMP to IMP is a simple deamination of the 6-amino group, while the conversion of GMP to IMP is a reductive step, requiring TPNH[33, 59]. The existence of these loops renders possible the interconversion of GMP and AMP if either one can be formed by the preformed pathways. Fig. 6.3 does not show the catabolic reactions that are competitive with the utilization of preformed purines in higher organisms but it should be noted that adenine and guanine are converted to hypoxanthine and xanthine respectively, while hypoxanthine is converted to xanthine and the latter is converted to uric acid.

Fig. 6.3 also brings out the fact that the two formylation steps in the de novo pathway are brought about by two different forms of the folic acid coenzyme group[10 XXVI]. The figure also includes the reaction by which AICAR is formed as a by-product of histidine

biosynthesis.   This intermediate is not necessarily an indicator of de novo purine synthesis since it can be formed in the presence of adenine under the proper conditions[57, 64].

The details of the Buchanan-Greenberg pathway and some proposed reaction mechanisms are discussed by Buchanan and Hartman[12] and by Buchanan et al[10] in references cited in Table 6.1.

It appears that the de novo pathway is widely distributed in nature.   Among the micro-organisms, there are many mutants that accumulate various intermediates in the latter stages of the de novo pathway[24].   An E. coli mutant that accumulates AIR was shown by Love and Levenberg[56] to be labeled by formate in the position corresponding to that shown in the schemes shown in Figs. 6.2 and 6.3.   Henderson and LePage[38] have demonstrated incorporation of glycine-2-C^{14} into purines of a variety of mouse tissues in vitro and in vivo and discuss the factors that influence the results. More papers of this type may be expected as the methods for testing for various intermediate steps are developed, and it will be interesting to note whether any of the intermediates between GAR and AICAR can be synthesized by pathways that are alternative to the ones shown, as well as to learn how the various tissues in higher organisms routinely balance their alternative routes of synthesis.

IV.   UTILIZATION OF PREFORMED PURINES

Prior to the discovery of the de novo pathway for the biosynthesis of the purine ribonucleotides it was known that many micro-organisms as well as higher organisms can utilize the purine bases and the purine ribonucleosides, although in the higher organisms, the utilization is very inefficient because of the rapid catabolism of these compounds, which appears to be more rapid in the case of guanine than in the case of

adenine[9]. Human cells grown in tissue cultures are
able to carry out de novo synthesis of purines but in
the presence of amethopterin (an antifolic acid,
Chapter XVII) this process is blocked and purines are
required, showing that they can be used[34]. Salzman
and Sebring[81] have shown that HeLa cells in tissue cul-
ture can utilize both glycine and preformed adenine
for purine synthesis and that just as in micro-organ-
isms, adenine prevents the utilization of glycine
(Chapter XII). Even in tissue culture, guanine was
poorly used because of rapid conversion to xanthine
relative to the conversion to GMP (Fig. 6.3)[81].

The actual pathways for utilizing the preformed bases
involve three different enzymes, corresponding to the
three reactions listed below, using adenine as the pu-
rine:

1.   Ribonucleoside phosphorylase:
      Adenine + ribose-1-phosphate $\rightleftharpoons$ adenosine +
          orthophosphate

2.   Ribonucleotide pyrophosphorylase:
      Adenine + 5'-phosphoribosylpyrophosphate
          (PRPP) $\rightleftharpoons$ adenosine 5'-phosphate +
          pyrophosphate

3.   Ribonucleoside phosphokinase:
      Adenosine + ATP $\rightleftharpoons$ adenosine 5'-phosphate + ADP

It may be seen that 2 can substitute for the combination
of 1 and 3 as a means of converting adenine to AMP
(Fig. 6.3).

The action of ribonucleoside phosphorylase was first
clarified by the classic studies of Kalckar[44] who work-
ed mainly with guanine and hypoxanthine. An enzyme
for adenine was obtained later from beef liver[10V]. Korn
and Buchanan[10VI] reported that all of the following bases
formed ribosides with a partially purified enzyme prep-
aration: xanthine, guanine, 5-amino-4-imidazole-
carboxamide (AICA), adenine, hypoxanthine, and 2,6-
diaminopurine.

The preceding reactions can lead to the formation of the 5'-ribonucleotides only in the presence of the appropriate ribonucleoside phosphokinase (reaction 3, above), and the significance of the ribonucleoside phosphorylase reaction (1, above) was somewhat diminished by the failure to find phosphokinases for all of the nucleosides that could be formed.   Apparently adenosine, [15, 51] 2-amino-adenosine[15] and AICA riboside[28] are the only nucleosides for which readily demonstrable phosphokinase activity has been found.   The adenosine phosphokinase was shown not to act on deoxyadenosine.

A new way of converting the preformed purines to the 5'-ribonucleotides was discovered by Kornberg, Lieberman and Simms in 1954 when PRPP[49] was discovered and shown to react as shown in 2, above.   The term ribonucleotide pyrophosphorylase[50] should be used as the name of a class of enzymes which include specific enzymes for various purines and pyrimidines. As early as 1952 Greenberg[28] as well as the Buchanan group[10VII] were studying the role of ribose phosphates in the de novo pathway of purine biosynthesis and established that there were two pathways for utilizing preformed purines.   In retrospect, it appears that they were prevented from isolating PRPP by the fact that this compound is labile enough to break down in the few minutes of heating or the acid employed to stop enzymic action.   This pathway for utilizing bases was shown to apply to adenine, guanine and hypoxanthine, but reaction with xanthine was not reported[50].   Reaction with AICA also occurs.

There has been a tendency to discount the importance of reactions 1 and 3, now that 2 has been discovered, but past experience has shown that in biological material, the existence of one pathway cannot justify the assumption that others do not exist, and the real question to be asked is in what cells and under what circumstances do the various pathways take precedence over one another.   This question is of great importance in chemotherapy that involves nucleic acid antimetab-

olites (Chapter XVII). Apparently pathways 1 and 3, or 2 assume great importance when preformed bases are available, and take precedence over the de novo pathway (Chapter XII).

V. PURINE DEOXYRIBONUCLEOTIDES

The biosynthesis of the purine deoxyribonucleotides is at present very poorly understood. It seems quite certain that there is no need for a parallel pathway of de novo synthesis comparable to the Buchanan-Greenberg pathway for the purine ribonucleotides. On the contrary it appears that the purine deoxyribonucleotides are formed from the ribonucleotides, and the unsettled question is whether the ribonucleotides are reduced as such or whether they are used as the source of free bases which then react with deoxyribose-1-phosphate. It seems entirely possible that both pathways occur. Since very little evidence for the direct reduction is available[30], the evidence for the conversion via the bases will be briefly mentioned. The key reaction, using either adenine or guanine as the base, is catalyzed by a purine deoxyribonucleoside phosphosphorylase:

Adenine + deoxyribose-1-phosphate ⇌ deoxyadenosine + orthophosphate.

This reaction has been observed in extracts of liver[18,20] as well as thymus and E. coli.[61] Deoxyribose-1-phosphate can be produced by a mutase[20, 61] acting on deoxyribose-5-phosphate, and the latter can be formed from glyceraldehyde-3-phosphate and acetaldehyde by an aldolase discovered by Racker[70]. More recently Boxer and Shonk[5] have suggested that the acetaldehyde may be replaced by threonine and an aldolase that yields the C_2 plus glycine.

It has been suggested that the purine deoxyribonucleoside phosphorylases may be identical with the ribonucleoside phosphorylases acting on the same bases, and there is a certain parallelism in distribution and

specificity.   While hypoxanthine, adenine and guanine are all converted to their deoxyribonucleosides, the reaction with xanthine is barely measurable[19].   It would be attractive to think that the nucleoside phosphorylases provide the mechanism for interchanging the bases with ribose and deoxyribose, but this reaction would have to be followed by the action of a deoxyribonucleoside phosphokinase to obtain the 5'-deoxyribonucleotides, and the enzymatic evidence for this step is not available.   In the case of adenosine phosphokinase, there was no action when deoxyadenosine was the substrate[51].

When it was discovered that bases could be converted directly to ribonucleotides by nucleotide pyrophosphorylase in the presence of PRPP, it was thought that perhaps an analogous deoxy-PRPP might be found but so far all attempts have been fruitless.

An enzyme called trans-N-deoxyribosylase has been studied in certain bacteria by MacNutt and by Roush and Betz[80].   Its action is to exchange the base moieties of purine and pyrimidine deoxyribosides without forming free deoxyribose-1-phosphate.   Studies by DeVerdier and Potter[16] described similar activity in rat liver but did not resolve the relation between this activity and that of the deoxyribonucleoside phosphorylases.

The whole question of the mode of formation of the purine deoxyribonucleotides is unresolved and in a much lower state of development than in the case of the pyrimidine deoxyribonucleotides (below) and even in the case of the pyrimidines the detailed mechanisms are not available.   There is convincing evidence that the pyrimidine ribonucleotides are reduced as such to the deoxyribonucleotides, and there is a possibility that these compounds may be used as a source of deoxyribose.   However, the present state of knowledge does not warrant the construction of a diagram to show how the purine deoxyribonucleotides arise.   That they do

arise and are phosphorylated to the triphosphate level and built into DNA will be shown in Chapter VII.

VI.   *HISTORICAL BACKGROUND FOR PYRIMIDINE BIOSYNTHESIS*

The biosynthesis of the pyrimidines has no history comparable to the pre-isotope or pre-microbiological era as in the case of the purines, probably owing to the fact that no compound comparable to uric acid was available for studies in higher organisms.

Early studies with isotopes were negative for the incorporation of isotopically-labeled uracil, thymine, and cytosine into nucleic acids in the rat.[9] In contrast, the nucleosides proved to be effective precursors and have been the subject of considerable study in terms of the preformed pathways,[9] with cytidine more effective than uridine. Studies on the de novo pathway of pyrimidine biosynthesis really began in 1944 with reports that orotic acid can replace pyrimidines as a growth factor for certain micro-organisms. Mitchell and Houlahan[62] reported in 1947 that in a series of Neurospora mutants the requirement for preformed pyrimidine could be satisfied with orotic acid and with oxalacetic acid and some related compounds. The use of isotopes for the study of pyrimidine synthesis in the rat began at this time in our laboratory with the synthesis of orotic acid-C^{14}, and similar studies were initiated in the Stockholm laboratories of Hammarsten and his collaborators, especially Reichard and Lagerqvist using N^{15}-labeled orotic acid. In 1949 this group reported extensive labeling of nucleic acid pyrimidines in nucleic acids in the rat[1]. Similar results were obtained by Hurlbert and Potter, who attacked the problem by comparing the acid soluble fraction with the acid insoluble fraction in rat livers at various times after the injection of orotic acid and showed a reciprocal relation between the amount of C^{14} in the two fractions over a period of 2 to 120 hours[41]. Attention

to the previously neglected pyrimidines of the acid soluble fraction led to the isolation of all of the pyrimidine nucleotides in the form of 5' mono-, di- and triphosphates[42], [83] and reaffirmed the proposal that this fraction contained the precursors of the nucleic acids[41].

The identification of the de novo precursors of the pyrimidines was hastened by the finding that ureido-succinic acid (= carbamylaspartic acid) can replace orotic acid for the growth of a bacterial mutant[88] and the finding that the same compound is incorporated into pyrimidines of nucleic acids in the rat[87].

The origin of carbamylaspartic acid from aspartic acid and carbamyl phosphate was clarified in 1954-56 by Reichard[71] and by Lowenstein and Cohen[58] and is well summarized in a review by Reichard[76] who also describes how the final pieces in the de novo synthesis of uridine-5-phosphate were added by Lieberman and Kornberg who showed that two steps between carbamyl aspartic and orotic acid involved the formation and oxidation of dihydro-orotic acid, while Lieberman, Kornberg and Simms[55] showed that orotic acid reacts with PRPP to form orotidine 5'-phosphate, the immediate precursor of uridine 5'-phosphate.

The pathway for the de novo synthesis of uridine 5'-phosphate from aspartic acid, CO_2 and NH_3 can now be taken as established, and all available information suggests that the other pyrimidine nucleotides are formed from UMP. Current studies fall into three categories: (1) The conversion of UMP to the other pyrimidine nucleotides (2) The role of the preformed pyrimidines and their nucleosides, and (3) The possibility of alternative de novo pathways for the synthesis of UMP[6], [63].

*VII. THE DE NOVO PATHWAY FOR THE BIOSYNTHESIS
       OF THE PYRIMIDINE RIBONUCLEOTIDES*

  *A.   The synthesis of orotic acid and its conversion to
        uridine-5'-monophosphate*

   Orotic acid is a key intermediate in the biosynthe-
   sis of the pyrimidine nucleotides. It is converted
   into 5'-UMP, and up to the present no pathway of
   de novo synthesis is known for any other pyrimi-
   dine: all of the other pyrimidines are either form-
   ed directly or indirectly from 5'-UMP or they are
   formed by "preformed" pathways of synthesis. As
   in the case of the purines, the direct products of
   the de novo synthetic pathway are the 5' monophos-
   phates, and not any of the higher phosphates or
   coenzymes, which are formed only as products of
   the monophosphates.

   It is convenient to discuss the synthesis of orotic
   acid and the sequence of reactions that lead to 5'-
   UMP by considering aspartic acid as the beginning
   of the synthetic pathway. Unpublished experiments
   in our laboratory have shown that the utilization of
   aspartic acid for pyrimidine biosynthesis was very
   poor in comparison with the utilization of the next
   compound in the series, which is carbamylaspartic
   acid. This suggests that possibly the series is
   controlled mainly at this point, and that any car-
   bamylaspartic acid that is formed is very likely
   to be converted all the way to 5'-uridylic acid.
   The intermediates in the conversion are shown in
   Fig. 6.4. The carbamyl phosphate for the first
   step shown in Fig. 6.4 can be formed either from
   CO_2, NH_3 and ATP or from citrulline and ATP or
   inorganic phosphate, according to Reichard[76], but
   apparently the first mentioned reaction is the most
   important. The conversion of aspartic acid to
   carbamylaspartic acid is for all practical purposes
   irreversible.
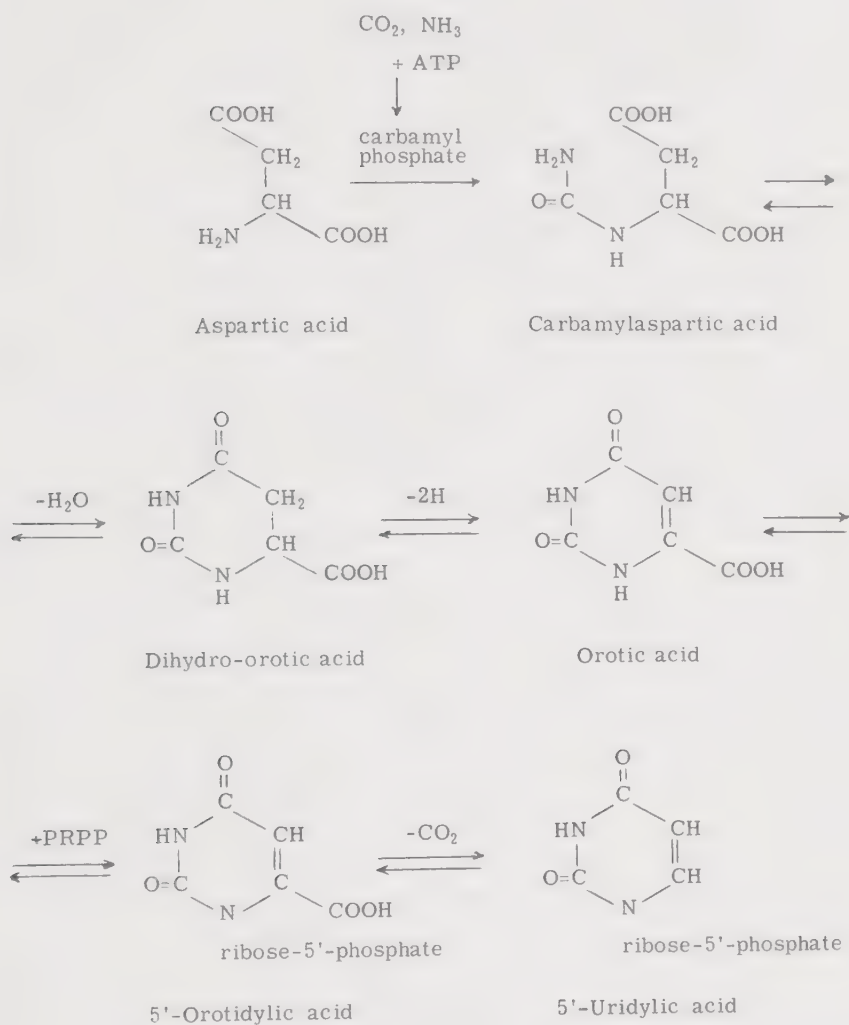
Fig. 6.4. The de novo synthesis of 5'-uridylic acid from aspartic acid, CO_2, and NH_3, via orotic acid. Carbamylaspartic acid has also been referred to as ureido-succinic acid but this term is no longer used.

A ring closure by loss of H_2O in a readily reversible step leads to the formation of dihydro-orotic acid and this compound is readily dehydrogenated

to form orotic acid. While DPN and TPN have been implicated in the reaction, it appears that their participation may be indirect[76,89]. There are many mutant micro-organisms that have genetic blocks at various points in the reaction sequence shown, and they excrete into the medium the compound that accumulates, for example orotic acid or dihydro-orotic acid. In 1959 a human case that appeared to have a congenital block in the metabolism of orotic acid was observed for the first time. A two year old child whose parents were second cousins was found to be excreting over a gram of orotic acid per day and to have an unusual type of anemia[39]. It was suggested that the pyrimidine metabolism was analogous to that of certain bacterial mutants previously studied by Yates and Pardee[90] since administration of pyrimidine nucleotides greatly reduced the orotic acid output in the child as well as in the mutant bacteria (Chapter XII).

When labeled orotic acid is injected into rats it is rapidly partitioned between the urine and the liver, with the proportion dependent upon the dose (unpublished) and in the liver it is phosphorylated and decarboxylated within 30-40 minutes, while the uridine nucleotides remain labeled for several days[42]. The liver is the major site of orotic acid uptake and the other organs take up very little, although the enzymes appear to be widely distributed[43].

The conversion of orotic acid to 5'-UMP proceeds via the formation of 5'-orotidylic acid by reaction with PRPP as the donor of the ribose-5-phosphate in a reaction analogous to the formation of the purine ribonucleotides[55]. The reaction is reversible and the enzyme is called orotidylic pyrophosphorylase. The next step is an irreversible enzymatic decarboxylation[55].

There have been no established enzymatic reactions involving any analogues of orotic acid passing through similar reactions to form cytidylic or thymidylic acid, and no evidence for the existence of higher phosphates of orotidylic acid. Thus we can say that the de novo pathway from aspartic acid leads to 5'UMP via orotic acid, with no known branches, at the present time (see below).

B. *Possible alternatives to the orotic pathway for de novo synthesis of 5'-UMP*

For several years Mokrasch and Grisolia have investigated the reactions related to the formation and possible utilization of dihydro-uracil and related compounds, and in 1959 reported that dihydrouridylic acid and carbamyl-β-alanine ribotide were better than orotic acid as precursors of uridine nucleotides as measured by incorporation into RNA in an enzyme system they employed[63]. If the decarboxylation of carbamylaspartic acid to carbamyl-β-alanine could proceed at a significant rate and if this compound could be converted to the ribotide, an alternative pathway to 5' UMP might be shown, but it is too early to evaluate the proposals at this time[76].

However, evidence from studies with Neurospora have shown that propionic acid and aminobutyric acid may be pyrimidine precursors in certain mutants[6]. These compounds might be convertible to β-alanine and to 5'-UMP via the above compounds although no intermediates have been suggested.

C. *Conversion of 5'-UMP to 5'-CMP*

It appears that there is no de novo pathway that leads to 5'-cytidylic acid: apparently the de novo pathway leads to 5'-uridylic acid, and this compound is converted in part to 5'-CMP, apparently

at the level of the triphosphate or diphosphate[54].
The situation is in some respects parallel to the
de novo synthesis of the purines, since the first
purine formed is a ribonucleoside monophosphate
in which there are no amino groups on the purine
ring; from this primary product, two amino de-
rivatives are formed, one a 6-amino (AMP) and
the other a 2-amino derivative (GMP).   In the
pyrimidine series, the first pyrimidine is also
without amino groups on the ring, (orotidylic acid),
and the 4-amino derivative (CMP) is analogous to
AMP.   However there is no known 2-amino deriva-
tive analogous to GMP and the conversion is not at
the monophosphate level, as in the case of the pu-
rines.

The conversion of UMP to CMP was indicated by
various metabolic studies in which the extent of
labeling of the nucleic acid pyrimidines was fol-
lowed as a function of time, after the injection of
a precursor.   Hecht and Potter[36] determined the
ratio of the specific activity of CMP from nuclear
RNA of rat liver to the specific activity of UMP
from the same samples after injection of orotic
acid and showed that the ratio extrapolated to zero
at zero time and approached 1.0 asymtotically at
about 24 hours.   Similar studies were repeated
with rat liver slices[37].

The question of the exact pathway from UMP to
CMP has been studied by Lieberman in extracts
from E. coli[54] and by Kammen and Hurlbert[45] using
high-speed supernatant fractions from homogenates
of the Novikoff hepatoma.   In both cases the con-
version appeared to occur at the level of UDP or
UTP but the systems differed in that the bacterial
system appeared to utilize NH_3 for the amination,
in an ATP-coupled reaction, while the mammalian
system required glutamine as the amino donor for
CTP production.   This difference in amination

systems apparently extends to the conversion of IMP to GMP since NH_3 appears to serve in bacterial systems while glutamine is required in mammalian systems[3]. However it should be remembered that ammonia plus glutamic acid can be converted to glutamine in the presence of ATP and glutamine synthetase, and the bacterial systems may have a system that is analogous to this reaction without requiring free glutamic acid.

The synthesis of the pyrimidine ribonucleotides for RNA and for coenzymes involves only two bases, uracil and cytosine, and it has been shown that uracil is built in the form of uridine 5'-monophosphate, while cytosine is formed when UDP or UTP is converted to CDP or CTP.

The pyrimidine deoxyribonucleotides can be formed directly from the ribonucleotides, as well as from the preformed bases and deoxyribosides, with a noteworthy limitation on the case of cytosine. We will first discuss the formation of the deoxyribonucleotides by an extension of the de novo pathway.

VIII. DE NOVO PATHWAY FOR THE BIOSYNTHESIS OF THE PYRIMIDINE DEOXYRIBONUCLEOTIDES

It was mentioned earlier in connection with the biosynthesis of the purine deoxyribonucleotides that attempts to find a deoxyribose analogue of PRPP had not been successful and it may be repeated here that there are no known reactions for the formation of the pyrimidine deoxyribonucleotides by pathways other than the prior de novo synthesis of 5'-uridylic acid, and the preformed pathways. Thus the material in this section involves mainly the conversion of 5'-UMP and 5'-CMP to the corresponding deoxyribonucleotides and the conversion of deoxyuridylic acid to thymidylic acid. It will be recalled that the small letter d will be used to indicate deoxy forms.

The important reactions are accordingly as follows:

(A) $5'\text{-UMP} \xrightarrow{\ 2H\ } 5'\text{-dUMP}$

(B) $5'\text{-CMP} \xrightarrow{\ 2H\ } 5'\text{-dCMP}$

(C) $5'\text{-dCMP} \xrightarrow{\ -NH_3\ } 5'\text{-dUMP}$

(D) $5'\text{-dUMP} \xrightarrow{\ +CH_3\ } 5'\text{-dTMP}$

It will be noted that of the three products formed, only dCMP and dTMP are utilized for DNA synthesis, while dUMP is an intermediate in the formation of dTMP.

These relationships are shown in their simplest form in Fig. 6.5, which is derived from a 3-dimensional diagram presented in 1956.[68] At that time it was pointed out that the reversibility of the steps or the existence of enzymes for each vector in the 3-dimensional diagram was unknown, and as recently as 1957 a hypothetical scheme[47] showed the two deaminations of Fig. 6.5 as aminations, with the arrows in the reverse direction. The "metabolic problems" shown in Fig. 6.5 are not necessarily solved in the simple way indicated, and the preceding section has mentioned that the conversion of UMP to CMP probably proceeds via UDP or UTP.
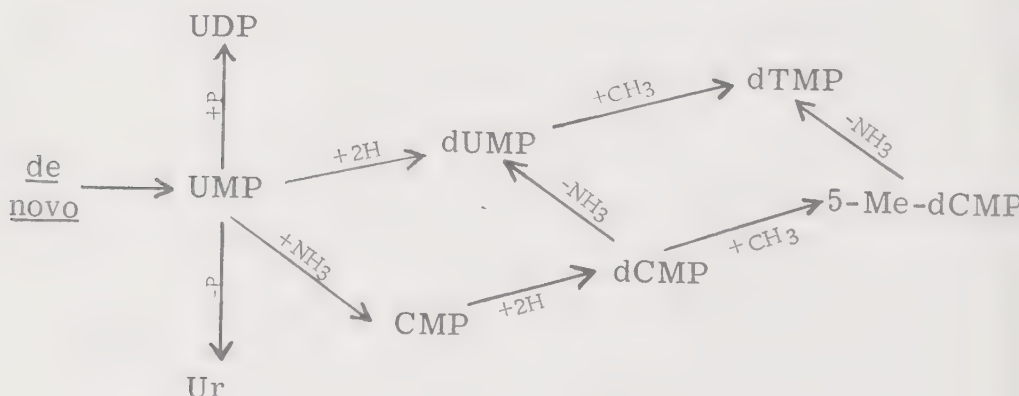
Fig. 6.5. Three-dimensional diagram illustrating regularities in the metabolic problems connected with the pyrimidine nucleotides

In addition to the above reactions, which suffice for the synthesis of most forms of DNA, there are two additional reactions involving the synthesis of special pyrimidine deoxyribonucleotides used for synthesis of DNA which contains 5-methyl-dCMP (in wheat germ DNA) and 5-hydroxy methyl-dCMP (in DNA of the T-even bacteriophages, Chapter XIII). These reactions stem from dCMP as follows:

(E) dCMP $\xrightarrow{\quad +CH_3 \quad}$ 5 Me-dCMP

(F) dCMP $\xrightarrow{\quad +CH_2OH \quad}$ 5 HM-dCMP
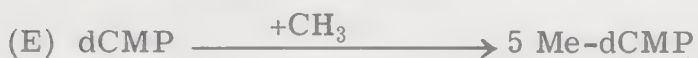
Reactions E and F will not be discussed in the present chapter.

All of the above reactions are included in a two-dimensional diagram Fig. 6.6 that shows the reactions of Fig. 6.5 plus additional reactions with which they must be integrated, including the preformed pathways and an indication of the over-all catabolic reactions in which uracil and thymine are converted to CO_2. The reactions numbered (A) to (F) above are similarly labeled in Fig. 6.6.

It is of considerable interest to find that 5'-deoxyuridylic acid can be formed in two different ways, either by the reduction of uridylic acid (Reaction A) or by the deamination of dCMP (Reaction C).

The three types of reactions shown in Fig. 6.5 will now be individually discussed.

A.   *The reductive steps*

The reduction of UMP to dUMP and of CMP to dCMP has been demonstrated in cell-free bacterial extracts.[30, 31, 77a] Reichard and Rutberg[77a] provided evidence favoring reduction at the diphosphate level when CMP was the compound added, and showed that TPNH could act as a reductant,

```
              RNA                           DNA
               ↗↖                            ↗↖
UDPX ↔ UTP → CTP ↔ CDPX    dCDPX ↔ dCTP    dTTP ↔ dTDPX
        ↕⇂    ↕⇂       "B"          ↕⇂        ↕⇂
       UDP   CDP ──────────────→ dCDP      dTDP
  "A"   ↕⇂    ↕⇂                   ↕⇂        ↕⇂          "A"
OMP → UMP   CMP                  dCMP   dTMP ← "D" dUMP
        ↕⇂    ↕⇂           "C"      ↕⇂        ↕⇂      "C"
  Or   Ur ← Cr                  Cdr Tr Tdr   Udr
   ↕    ↑                          ↕    ↑     ↕
   O    U    C                     C    T     U
   ↑    ↑                               ↓
  DHO  DHU                            DHT
   ↑    ↑                               ↓
  CA   BUP                            BUIB
   ↑    ↓                               ↓
  Asp  BAL                            BAIB
```
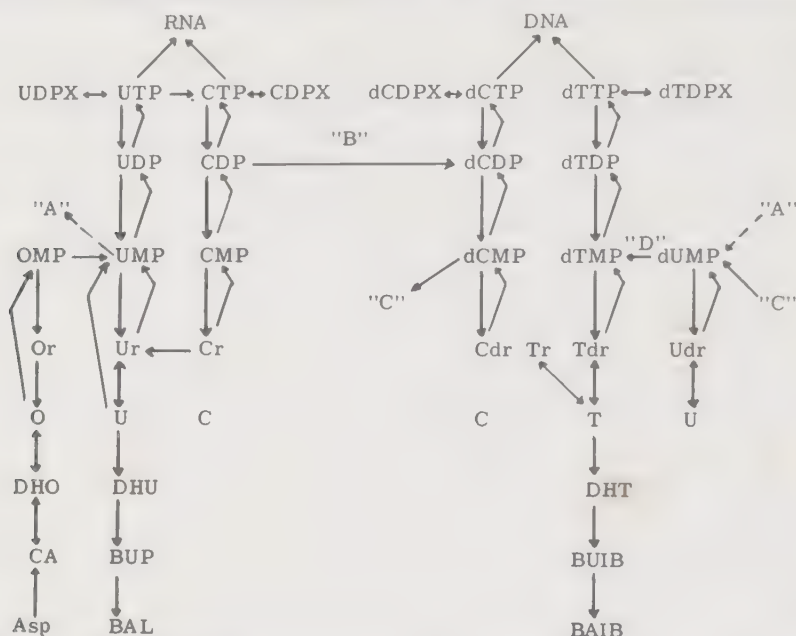
Fig. 6.6. Integration of the de novo pathway of pyrimidine ribonucleotide biosynthesis (Fig. 6.4) with preformed pathways, interconversions, formation of deoxyribonucleotides, and catabolic pathways. The letter d is used to indicate deoxy- in all cases, including the thymidine phosphates, which now have to be so designated because of the thymine riboside (Tr). The letter r is used to indicate ribosides, and dr to indicate deoxyribosides. DH indicates dihydro-, CA = carbamylaspartic, Asp= aspartic; The X substituents are discussed in Chapter IX and the catabolic pathways (BUP, BAL, BUIB, and BAIB) are discussed in Chapter VIII. The bent arrows are used to indicate that the reaction is brought about by means of a different enzyme as in Chapter VIII. Reactions "A", "B", "C", "D" described in the text.

while DPNH was relatively ineffective. The problem has also been studied by Reichard with minced chick embryo[75]. Deoxycytidine appeared to be a metabolic "dead-end" and not an essential intermediate in the conversion of cytidine to dCMP. This suggestion is followed in Fig. 6.6. This conclusion is also supported by the experiments of Hecht and Potter using regenerating rat liver[36]. The latter studies also provided in vivo evidence that there is a direct pathway from UMP to dTMP by showing that the ratio of the specific activity of dCMP from DNA to the specific activity of dTMP from the same samples of DNA approaches zero at zero time after injection of orotic acid.[36] This result was not obtained in slices of regenerating

liver, where the ratio was nearly the same at 1, 2, and 4 hours of incubation.

Another type of evidence suggesting a direct reduction of a cytosine ribonucleotide to a cytosine deoxyribonucleotide is based on the injection of cytidine-C^{14} labeled in both the pyrimidine and the ribose moiety. Rose and Schweigert[79] showed that the ratio of C^{14} in cytosine to C^{14} in the sugar remained the same in the dCMP of DNA as in the cytidine injected, which was taken to mean that reduction occurred without separation of cytosine from the sugar moiety. A similar conclusion had been reached earlier by Hammarsten, Reichard and Saluste[35] on the basis of the fact that free cytosine or uracil is poorly utilized by the rat for nucleic acid synthesis while cytidine and uridine are utilized fairly well. The Rose and Schweigert experiments[79] have now been repeated with uridylic and cytidylic acids[78] and with uridine[74]. These data do not exclude the possibility of a reduction at the level of CDP-choline as suggested by Kennedy et al.[45a] The question of whether all tissues and organisms carry out reductions of both uridine and cytidine ribonucleotides is at present completely open. The fact that some tissues can make dUMP from dCMP (next section) while some cannot suggests that the latter tissues would have a greater need for the ability to reduce UMP to dUMP.

B.   *The deaminative steps*

The deamination of dCMP to dUMP (Reaction C) and of 5-Me-dCMP to TMP was reported for sea urchin eggs by Scarano[82]. The deamination of dCMP to dUMP by extracts of embryonic rat liver and adult rat thymus, bone marrow, and Novikoff hepatoma was reported by Maley and Maley[60]. The enzyme was later reported to be absent from the Dunning hepatoma but present in the livers of

rats with rapid bile duct proliferation[67]   (see Chapter XVI).   The enzyme appears to be correlated with DNA synthesis but it is apparently not required by systems that can obtain dUMP by another route.

C. *The methylation steps*

Most studies on the methylation step have centered around the conversion of dUMP to dTMP (Reaction D) and it is apparently established that the conversion occurs as written and not at the deoxyriboside or polyphosphate levels. Indeed, there appears to be no enzymes[21] available for the conversion of dUMP to the higher phosphates (Fig. 6.6). There has been considerable evidence to show that the methyl group came from the C_1 donors via a folic acid coenzyme but both the acceptor and the donor were unknown. Recent studies[21, 40] indicate that dUMP is indeed the acceptor and suggest that the folic acid coenzyme is N^{10}-hydroxymethyltetrahydrofolic acid. Bolinder and Reichard[3] have suggested that vitamin B_{12} may be involved in the formation of the hydroxymethyl coenzyme from another form of the folic coenzyme. Whether similar donors are involved in the conversions of dCMP shown by reactions (E) and (F) is not yet clear.

IX.   *UTILIZATION OF PREFORMED PYRIMIDINES*

A. *Cytosine, cytidine and deoxycytidine*

In the utilization of preformed pyrimidines the position of cytosine is at present unexplained and it stands more or less isolated as in Fig. 6.6. Apparently it reacts neither with ribose-1-phosphate nor with deoxyribose-1-phosphate[47] nor will it exchange with thymine or uracil in nucleoside

combination in extracts that are active with the other pyrimidines.[16] Similarly, the reaction of cytosine with PRPP has not been observed in extracts of certain organisms that will utilize uracil even though these extracts contain the enzyme for utilizing uracil in the PRPP reaction.[47]

One has the impression that in the animal body at least, the system is arranged so as to conserve cytidine and deoxycytidine, which can only be degraded to the extent that they can be converted to uridine and deoxyuridine (Fig. 6.6).

It was reported that Cdr was not deaminated directly to Udr but that the deamination occurred only after conversion to dUMP as shown in Fig. 6.6, and even this step occurred in only a few organs.[60] On the other hand, the deamination of cytidine probably occurs at the nucleoside level[32]. Since cytidine and deoxycytidine cannot be converted to cytosine, the converse is also true and as a result cytosine is an extremely poor precursor of the pyrimidines and inferior to uracil, while cytidine is much better than uridine.[10] Deoxycytidine is utilized for both the cytosine and thymine of DNA but was not utilized for RNA.[77] This is further evidence that deoxycytidine does not get converted to cytidine or to CMP to a significant extent, while it does get converted in part to TMP via dCMP and dUMP (Fig. 6.6). Experiments with the cytidylic acids[78] suggest that the phosphates are hydrolyzed off and the cytidine moiety is reutilized without separation of base and sugar.

B. *Uracil, uridine, and deoxyuridine*

Although uracil is rapidly catabolized by the liver, it can be converted to uridine by uridine phosphorylase[13, 16] and this can be converted to 5'-UMP by a uridine phosphokinase[13]. Although a uridylic

pyrophosphorylase yielding uracil and PRPP was found in certain bacteria[47] it does not appear to be in liver[13]. Uracil can be utilized if present at extremely high levels if the degradative pathway is saturated[14]. These experiments were carried out with rat liver slices. In the whole animal very little uracil can be utilized for nucleic acid synthesis because of the rapid breakdown in the liver. Uridine is utilized much better than uracil and shows the same effect of concentration. At low levels most of it is converted to uracil by uridine phosphorylase and thus catabolized[14]. Thus the same three enzymes that were described for adenine in the purine section have been shown for uracil, although it appears that the direct conversion of uracil to 5'-UMP has been observed only in bacteria[13, 47].

The following scheme shows this portion of Fig. 6.6 in greater detail:
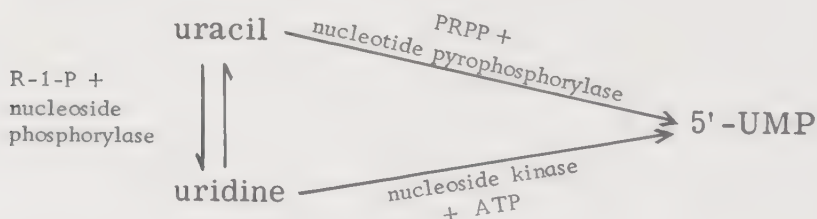
Coming now to deoxyuridine, we find that this compound can be converted to uracil by a deoxyribonucleoside phosphorylase[16] or to 5'-dUMP by a kinase.[48] Deoxyuridine can serve as a precursor for the thymidylic acid of DNA[22, 73] proceeding by way of 5'-dUMP[21] (see previous section IX, C) which cannot be converted to higher phosphates.[21] The latter fact is believed to account for the absence of dUMP from DNA. Experiments with dUTP prepared chemically from dCTP showed that the DNA polymerase does not reject the deoxyuridine triphosphate, and if it were formed in cells it would probably be incorporated into DNA.

C. Thymine, thymine riboside, and thymidine

Like uracil, thymine is an extremely poor precursor and no data on its incorporation into DNA in whole animal experiments has been reported, although thymine is vigorously converted to thymine riboside or to thymidine in the presence of uridine or deoxyuridine[16]. The concentration of these compounds is apparently quite low in vivo, while the capacity of the liver to catabolize thymine is very high (Chapter VIII). The role of thymine riboside is at present not clear. Thymidine is an effective precursor of DNA thymidylic acid[23, 65, 77], although it is rapidly converted to thymine, which is catabolized by the liver within 1-2 hours after injection (Morse and Potter unpublished). During this time it is also converted to TMP by thymidine kinase and thence to TTP, which is utilized for DNA synthesis. Thus the period of DNA labeling by thymidine is very brief[65] but this is a useful property when thymidine is used for autoradiographic studies of DNA synthesis. Bollum and Potter[4] have shown marked increases in thymidine kinase in regenerating rat liver at the time of maximum DNA synthesis, while normal liver contained almost none of the enzyme. Thus the enzymatic pathways for thymine and thymidine are as shown in Fig. 6.6 and no conversion of thymine to dTMP by a direct route that by-passes Tdr is known.

X. ALTERNATIVE METABOLIC PATHWAYS

One of the principles that needs to be emphasized in a presentation of this type is that life processes seem to be built up in terms of metabolic pathways that have many branches. We have previously discussed this fact, emphasizing that there are multiple coexistent alternative pathways for both anabolic and for catabolic processes.[69] Any given compound can be thought of in terms of the convergent pathways that lead to its for-

mation and the divergent pathways that lead to its removal. The divergent pathways may include both synthetic and degradative pathways and the ultimate rate of synthesis will depend upon the competition between these pathways. In higher organisms, the distribution of the synthetic and degradative enzymes varies from one tissue to another, and we have seen that the liver is one of the more important sites of pyrimidine and purine catabolism, exerting an effect that would seem to be opposed to nucleic acid synthesis in the rest of the body (Chapter VIII). Many examples of converging and diverging pathways have been presented in Figs. 6.3 (purines) and 6.6 (pyrimidines) and the student should understand that these reactions are not all present to the same extent in any one cell, or in any one compartment of a given cell type. These figures represent only the beginnings of our understanding of these complex metabolic networks and they will have to be revised in terms of new reactions and demonstrations of reversibility or near irreversibility, and in terms of distribution and control mechanisms. The existence of multiple alternative pathways is one of the things that greatly complicates chemotherapy (Chapter XVII), and the understanding of the cancer problem (Chapter XVI), and there are thus urgent practical reasons for attempting to organize existing knowledge in order to facilitate the understanding of contemporary literature in this field.

REFERENCES

1. ARVIDSON, ELIASSON, HAMMARSTEN, REICHARD, von UBISCH, and BERGSTRÖM. J. Biol. Chem. 179, 169 (1949).
2. BESSMAN, LEHMAN, ADLER, ZIMMERMAN, SIMMS and KORNBERG. Proc. Nat. Acad. Sci. (U.S.) 44:633 (1958).
3. BOLINDER and REICHARD. J. Biol. Chem. 234:2723 (1959).
4. BOLLUM and POTTER. Cancer Research 19:561 (1959).
5. BOXER and SHONK. J. Biol. Chem. 233:535 (1958).
6. BOYD and FAIRLEY. J. Biol. Chem. 234:3232 (1959).
7. BRANDENBERGER. Helv. Chim. Acta. 37:641 (1954) and Biochim. Biophys. Acta. 15:108 (1954).
8. BRANDENBERGER and BRANDENBERGER. Helv. Chim. Acta. 37:2207 (1954).
9. BROWN and ROLL in Chargaff and Davidson 2:341 (1954).
10. BUCHANAN et al. see Table 6.1. Each reference citation in the text will specify the paper number as given in the table.
11. BUCHANAN, FLAKS, HARTMAN, LEVENBERG, LUKENS and WARREN in Wolstenholme and O'Connor, editors. The Chemistry and Biology of the Purines, Churchill, London, 1957, p. 233.
12. BUCHANAN and HARTMAN. Adv. Enzymol. 21:200 (1959).
13. CANELLAKIS. J. Biol. Chem. 227:329 (1957).
14. CANELLAKIS. J. Biol. Chem. 227:701 (1957).
15. CAPUTTO. J. Biol. Chem. 189:801 (1951).
16. De VERDIER and POTTER. J. Nat. Cancer Inst. 24:13 (1960).
17. EDSON, KREBS and MODEL. Biochem. J. 30:1380 (1936).
18. FRIEDKIN. J. Biol. Chem. 184:449 (1950).
19. FRIEDKIN. J. Am. Chem. Soc. 74:112 (1952).
20. FRIEDKIN and KALCKAR. J. Biol. Chem. 184:437 (1950).

21. FRIEDKIN and KORNBERG in McElroy and Glass. The
     Chemical Basis of Heredity. Johns Hopkins Press,
     Baltimore 1957, p. 609.
22. FRIEDKIN and ROBERTS. J. Biol. Chem. 220:653
     (1956).
23. FRIEDKIN, TILSON and ROBERTS. J. Biol. Chem.
     220:627 (1956).
24. FRIEDMAN and MOAT. Arch. of Biochem. Biophys.
     78:146-156 (1958).
25. GOTS. Nature 172:256 (1953).
26. GREENBERG. Arch. Biochem. 19:337 (1948).
27. GREENBERG. J. Biol. Chem. 190:611 (1951).
28. GREENBERG. J. Biol. Chem. 219:423 (1956).
29. GREENBERG and SPILMAN. J. Biol. Chem. 219:411
     (1956).
30. GROSSMAN. Fed. Proc. 17:235 (1958).
31. GROSSMAN and HAWKINS. Biochim. Biophys. Acta
     26:657 (1957).
32. GROSSMAN and VISSER. J. Biol. Chem. 209:447
     (1954).
33. GUARINO and YUREGIR. Biochim. Biophys. Acta
     36:157 (1959).
34. HAKALA and TAYLOR. J. Biol. Chem. 234:126
     (1959).
35. HAMMARSTEN, REICHARD and SALUSTE. J. Biol.
     Chem. 183:105 (1950).
36. HECHT and POTTER. Cancer Research 16:999 (1956).
37. HECHT and POTTER. Cancer Research 18:186 (1958).
38. HENDERSON and LePAGE. J. Biol. Chem. 234:2364
     (1959).
39. HUGULEY, BAIN, RIVERS, and SCOGGINS. Blood,
     14:615 (1959).
40. HUMPHREYS and GREENBERG. Arch. Biochem.
     Biophys. 78:275 (1958).
41. HURLBERT and POTTER. J. Biol. Chem. 195:257
     (1952).
42. HURLBERT and POTTER. J. Biol. Chem. 209:1
     (1954).
43. HURLBERT and REICHARD. Acta Chem. Scand.
     9:251 (1955).

44.   KALCKAR. J. Biol. Chem. 158:723 (1945), 167:477
         (1947).
45.   KAMMEN and HURLBERT. Cancer Research 19:654
         (1959).
45a. KENNEDY, BORKENHAGEN, FENCIL, and SMITH.
         J. Biol. Chem. 234:1998 (1959).
46.   KNIGHT. Vitamins and Hormones 3:105 (1945).
47.   KORNBERG in McElroy and Glass. The Chemical
         Basis of Heredity Johns Hopkins Press, Baltimore
         1957, p. 579.
48.   KORNBERG, LEHMAN, BESSMAN and SIMMS. Bio-
         chim. Biophys. Acta 21:197 (1956).
49.   KORNBERG, LIEBERMAN and SIMMS. J. Biol. Chem.
         215:389 (1955).
50.   KORNBERG, LIEBERMAN and SIMMS. J. Biol. Chem.
         215:417 (1955).
51.   KORNBERG and PRICER. J. Biol. Chem. 193:481
         (1951).
52.   KREBS and HENSELEIT. Z. Physiol. Chem. 210:33
         (1932).
53.   LAGERQVIST. J. Biol. Chem. 233:143 (1958).
54.   LIEBERMAN. J. Biol. Chem. 222:765 (1956).
55.   LIEBERMAN, KORNBERG, and SIMMS. J. Biol. Chem.
         215:403 (1955).
56.   LOVE and LEVENBERG. Biochim. Biophys. Acta
         35:367 (1959).
57.   LOVE and BOYLES. Biochim. Biophys. Acta 35:374
         (1959).
58.   LOWENSTEIN and COHEN. J. Biol. Chem. 213:689
         (1955) and 220:57 (1956).
59.   MAGER and MAGASANIK. J. Biol. Chem. 235:1474
         (1960).
60.   MALEY and MALEY. J. Biol. Chem. 234:2975 (1959).
61.   MANSON and LAMPEN. J. Biol. Chem. 191:95 (1951)
         and 193:539 (1951).
62.   MITCHELL and HOULAHAN. Fed. Proc. 6:506 (1947).
63.   MOKRASCH and GRISOLIA. Biochim. Biophys. Acta
         34:165 (1959).
64.   MOYED and MAGASANIK. J. Am. Chem. Soc. 79:4812
         (1957).
65.   NYGAARD and POTTER. Rad. Res. 10:462 (1959).

66.   ORSTROM, ORSTROM and KREBS. Biochem. J. 33: 990 (1939).
67.   PITOT and POTTER. Biochim. Biophys. Acta. 40:537 (1960).
68.   POTTER, V.R. Texas Rpts. Biol. and Med. (1956).
69.   POTTER and HEIDELBERGER. Physiol. Reviews 30:487 (1950).
70.   RACKER. J. Biol. Chem. 196:347 (1952).
71.   REICHARD. Acta Chem. Scand. 8:795, 1102 (1954).
72.   REICHARD in Chargaff and Davidson 2:277 (1954).
73.   REICHARD. Acta Chem. Scand. 9:1275 (1955).
74.   REICHARD. Acta Chem. Scand. 11:11 (1957).
75.   REICHARD. Biochim. Biophys. Acta. 27:434 (1958).
76.   REICHARD. Adv. Enzymol. 21:263 (1959).
77.   REICHARD and ESTBORN. J. Biol. Chem. 188:839 (1951).
77a. REICHARD and RUTBERG. Biochim. Biophys. Acta 37:554 (1960).
78.   ROLL, WEINFELD and CARROLL. J. Biol. Chem. 220:455 (1956).
79.   ROSE and SCHWEIGERT. J. Biol. Chem. 202:635 (1953).
80.   ROUSH and BETZ. J. Biol. Chem. 233:261 (1958).
81.   SALZMAN and SEBRING. Arch. Biochem. Biophys. 84:143 (1959).
82.   SCARANO. Biochim. Biophys. Acta 29:459 (1958); J. Biol. Chem. 235:706 (1960).
83.   SCHMITZ, HURLBERT and POTTER. J. Biol. Chem. 209:41 (1954).
84.   SCHULER and REINDEL. Z. Physiol. Chem. 221:209 (1933).
85.   SHIVE, ACKERMANN, GORDON, GETZENDANER and EAKIN. J. Am. Chem. Soc. 69:725 (1947).
86.   STETTEN and FOX. J. Biol. Chem. 161:333 (1945).
87.   WEED and WILSON. J. Biol. Chem. 207:439 (1954).
88.   WRIGHT, MILLER, SKEGGS, HUFF, WEED and WILSON. J. Am. Chem. Soc. 73:1898 (1951).
89.   WU and WILSON. J. Biol. Chem. 223:195 (1956).
90.   YATES and PARDEE. J. Biol. Chem. 221:757 (1956).

Chapter VII
Biosynthesis of the
Nucleic Acids

Chapter VII
Biosynthesis of the
Nucleic Acids

I.   *INTRODUCTION*

The biosynthesis of the nucleic acids in cell-free solu-
ble enzyme systems is a very recent development. In
1955 Grunberg-Manago and Ochoa[22] discovered an
enzyme for the synthesis of RNA in bacteria; in 1956
Kornberg, Lehman, Bessman, and Simms[39a] discov-
ered a DNA-synthesizing system in bacteria; and in
1957 Bollum and Potter[10] reported the occurrence of a
similar DNA-synthesizing system in extracts from
regenerating rat liver. None of these events was re-
corded in time to be included in volumes I or II of the
Chargaff and Davidson series, published in 1955, but
an excellent account of these discoveries is given by
Khorana[40] in volume III of this series and with 119
references it constitutes a more comprehensive review
than will be attempted here. The discovery of all of
the ribonucleotide components of ribonucleic acid in
the form of the 5' mono-, di-, and triphosphates in the
acid soluble fraction of a variety of animal tissues[57]
was reported in 1954 but not in time to affect the main
discussions in the chapters by Reichard, by Schlenk,
by Brown and Roll, or by Smellie in volume II of
Chargaff and Davidson, although the finding was men-
tioned in several of the addenda: Schlenk[56] pointed out
that the newly isolated ribonucleoside-5'-triphosphates
"may occupy a key position in future concepts of poly-
nucleotide synthesis", and Smellie[63] noted the correla-
tion between the specific activity of 5'-UMP in the acid
soluble fraction of rat liver and the specific activity of

uridylic acid in the nuclear RNA of the same samples
at various times after injection of orotic acid-C^{14}, as
reported by Hurlbert and Potter,[38] who had previously
shown a very close precursor-product relationship be-
tween the radioactivity in the acid-soluble fraction and
the RNA of the rat livers,[37] but had not identified the
radioactive intermediates, which they later accounted
for as pyrimidine ribonucleotides.

The timing of the developments in this field is men-
tioned to enable the student to read the valuable re-
views of whole animal experiments in earlier reference
books with a perspective that will enable him to inter-
pret some of the conjectures that were made at that
time.

In the discussion to follow, it will be useful to think of
nucleic acid synthesis in terms of the replication of a
pre-existing pattern or code expressed as a sequence
of bases attached to a backbone of alternating sugar-
phosphate linkages. This replication of pattern prob-
ably accounts for the fact that much of the current work
in the biosynthesis of the nucleic acids involves the role
of the "primer", which is the term used to describe an
oligo- or polynucleotide that can be shown to be nec-
essary for nucleic acid synthesis in addition to sub-
strates and enzyme. The structure of the nucleic acids
was described in Chapter III, and the notation used to
describe short oligonucleotides was given at that point.
Here it will be sufficient to repeat that in such an oligo-
nucleotide, for example, pGpCpA, the p on the left
indicates a 5' monoesterified phosphate and the A on
the right indicates an adenosine with 2'OH and 3'OH
unesterified. In the case of deoxyribonucleotides a d
at the beginning is intended to apply to the whole series
to the right unless otherwise specified. In the case of
double-stranded segments a modified notation is need-
ed, and this is shown in Fig. 7.1.

I. Model for DNA synthesis incorporating primer (dpTpTpT).

```
G-dr-ppp                /
    +               G-dr-p
    /                   /
T-dr-p              T-dr-p
    /                   /
T-dr-p    ⟶         T-dr-p            +  pyrophosphate
          ⟵             /
    /               
T-dr-p              T-dr-p
```

II. Model for DNA synthesis not incorporating primer (dpTpTpT).

```
        /                           /
ppp-dr-A...T-dr-p       ppp-dr-A...T-dr-p
                            /           /
ppp-dr-A...T-dr-p  ⟶    p-dr-A...T-dr-p     +  2 pyrophosphate
                   ⟵        /           /
ppp-dr-A...T-dr-p       p-dr-A...T-dr-p
                            /
```
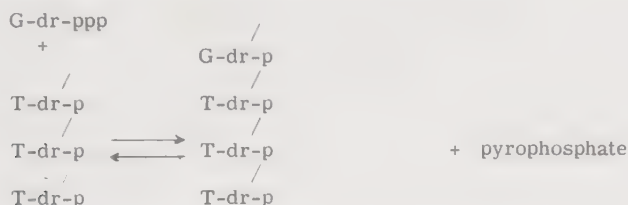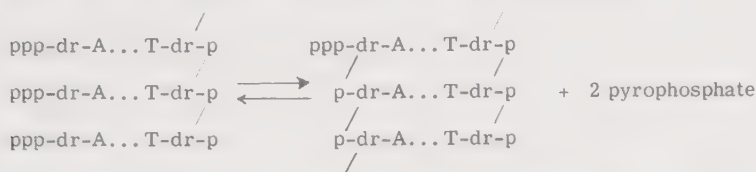
III. Model for RNA synthesis incorporating primer with 3'OH (pApApA).

```
A-r-pp                  /
   +                A-r-p
   /                    /
A-r-p    ⟶          A-r-p
   /     ⟵              /
A-r-p               A-r-p              +  inorganic phosphate

A-r-p               A-r-p
```
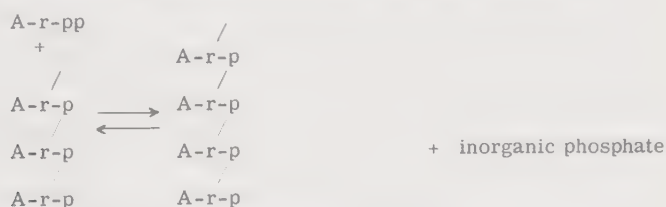
IV. Model for RNA synthesis not incorporating primer with 3'P (pApApAp).

```
        p                           p
        /                           /
pp-r-A...A-r-p          pp-r-A...A-r-p
                            /           /
pp-r-A...A-r-p  ⟶       p-r-A...A-r-p       +  2 inorganic phosphate
                ⟵           /           /
pp-r-A...A-r-p          p-r-A...A-r-p
                            /
```
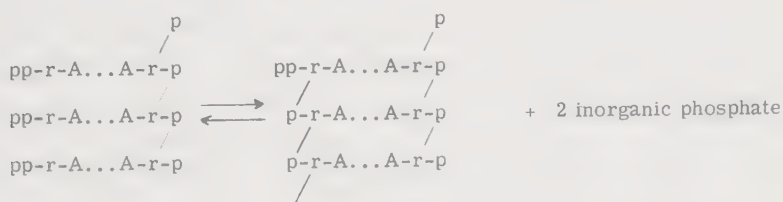
Fig. 7.1. Abbreviated oligonucleotide and mononucleotide formulae arranged to facilitate discussions involving primer function and the role of hydrogen bonding between bases in paired strands. The 5'-phosphates (p), -diphosphates (pp), and -triphosphates (ppp) are written to the left or right of the nucleosides. The 3'-phosphates are written above or below and diagonally from the symbols for ribose (r) or deoxyribose (dr), and 3' OH are shown only on polymers, as a diagonal without attached (p). It seems likely that metal ions (not shown) may be involved in some of the inter-strand bonding. It should be noted that the letters A, G, U, C, and T represent bases in the proposed vertical formulae and nucleosides in the horizontal shorthand formulae.

Another point that should be emphasized is the fact that the existence of "transfer-RNA" in the soluble fraction of the cytoplasm and its role in protein synthesis was completely unanticipated when volumes I and II of Chargaff and Davidson's series was written, and most of the studies on incorporation of precursors into RNA in multiple enzyme systems or in vivo have not distinguished between the synthesis of RNA that might conceivably be based on a template and RNA that is for the purpose of catalyzing the transfer of amino acids (Chapter XI).

Finally it may be noted that the first reports of net synthesis of nucleic acids have been based on studies with bacterial extracts[22, 39a]. These reports will be described in separate sections before discussing studies with animal tissues, since they are helpful in interpreting the latter. It is of interest that the studies on bacterial extracts have consistently shown the ribonucleoside-5'-diphosphates to be the substrates and inorganic phosphate to be one of the products in the synthesis of RNA[22], while the deoxyribonucleoside-5'-triphosphates are the substrates and inorganic pyrophosphate is one of the products in the synthesis of DNA[39a]. More recent work with animal tissues has indicated that the ribonucleoside-5'-triphosphates are used as substrates in certain reactions involving an increase in the length of the RNA chain but it is not clear whether this is in all cases limited to the soluble or transfer type of RNA.[30]

II.   BIOSYNTHESIS OF RIBONUCLEIC ACID

A.   Polynucleotide phosphorylase from bacteria

The earliest studies on polynucleotide phosphorylase established that the substrates are ribonucleoside-5'-diphosphates, that the product is structurally analogous to native RNA, and that inorganic phosphate is a stoichiometric product cor-

responding to the number of moles of nucleoside diphosphate used up. The original experiments were carried out with extracts of Azotobacter vinelandii by Ochoa and coworkers[22, 45] and these were soon followed by confirming reports by Littauer and Kornberg[41] using E. coli and by Beers[3] using Micrococcus lysodeikticus.

The reaction catalyzed is as follows:

$$n \ ppB \rightleftharpoons (pB)_n + n \ P \tag{1}$$

in which B is used to mean either a purine or a pyrimidine riboside. The reaction is completely reversible and a polyribonucleotide can be phosphorolyzed with inorganic phosphate to yield the ribonucleoside-5'-diphosphates of all the purines and pyrimidines in the original RNA. The reaction equilibrium is affected mainly by the ratio of inorganic phosphate to ribonucleoside diphosphate and in the conversion of the latter to RNA the equilibrium is reached when 60 to 80 per cent of the substrate has reacted. The data suggest that the 3'-5' phosphate diester linkage has a free energy of hydrolysis that is about as great as that of the pyrophosphate in the ribonucleoside-5'-diphosphates. The enzyme requires magnesium ions and as in many other nucleotide systems the magnesium requirement is related to the amount of substrate present. The affinity of the enzyme for the diphosphates is quite low,[41] which would make the inorganic phosphate concentration a very critical factor in RNA synthesis if this enzyme were responsible for events in vivo.

One of the more interesting findings has been the fact that polymers of only one type of ribonucleotide have been formed in the presence of the enzyme. Apparently it has been possible to form polymers from ADP, IDP, CDP, and UDP quite readily[3, 41, 45, 61, 62] and from GDP only with primer

present[62]. The products are referred to as poly
A, poly I, poly C, poly U, and poly G, respective-
ly. The latter has not been obtained in quantity[45,62]
and poly I has been used in its place for experi-
ments involving hydrogen bonding. A poly T has
been synthesized from synthetic thymine riboside-
5'-diphosphate[21]. It is not clear whether a single
enzyme acts on all of the diphosphates or on any
mixture of them or whether a specific enzyme is
needed for each one as suggested by Olmstead.[46]
Discussion of the mechanism of action of the syn-
thetic reaction and of the phosphorolysis involves
the function of primers, the action of which is not
included in equation (1) above.

1. Primer function. The original studies gave
    no indication that primers were necessary for
    RNA synthesis[3, 23, 41] but subsequent studies
    by Mii and Ochoa[43] showed a lag period which
    could be eliminated by adding poly A when
    ADP was the substrate or by poly U when UDP
    was the substrate. Poly U was ineffective
    with ADP and poly A was ineffective with UDP.
    A number of similar and more complex rela-
    tionships were shown with CDP.

    Polymerization by adding to the 3' end of the
    primer has been clearly shown with short
    oligonucleotides as primers.[61] These studies
    have been facilitated by the use of pancreatic
    ribonuclease as discussed in Chapter III. The
    smallest compound that will serve as a primer
    is a 3', 5' phosphodiester, for example ApA.
    In addition pApA, pApApA, and pApApApA are
    effective, as well as the corresponding com-
    pounds without the 5' monophosphate end
    groups: ApA and ApApA. Such compounds
    could serve as primers for UDP, in which
    case the product consisted of primer joined
    to poly U. Thus for example

$$\text{pApA} + \underline{n}\text{UDP} \rightleftharpoons \text{pApA(pU)}_{\underline{n}} + \underline{n}\text{P} \qquad (2)$$

Since pancreatic ribonuclease can split UpA or UpU but not ApU diesters (Chapter III), the above product treated with this enzyme will undergo the following reaction:

$$pApA(pU)\underline{n} \rightarrow pApAUp+(\underline{n}-2)Up + U \quad (3)$$

which is taken to prove that the product in reaction (2) was as formulated. The oligonucleotide product in (3) is terminated by a 3' phosphate and is useful for further studies on primer function[61]. The above studies show that the 3'OH primers are incorporated into the final product. When the oligonucleotide is terminated by a 3' phosphate it may serve as a primer in the sense of abolishing the lag period but the new polynucleotide is not attached to the primer[61]. This raises the question of whether the 3'-phosphate primers act as templates for hydrogen-bonding the mononucleotides long enough to facilitate their polymerization into a new oligonucleotide with a free 3'-OH. This mode of action would be analogous to the action of the DNA primers, which will be discussed later. It might also account for the effects of magnesium ions and other cations, which might be expected to determine the kind and extent of hydrogen bonding between bases. The two types of primer function are illustrated in Fig. 7.1, III and IV.

2. Phosphorolysis. Extensive studies of the phosphorolytic splitting of oligoribonucleotides by the Ochoa enzyme have been carried out by Singer[60]. The results show that the presence of a 3'-phosphate end group blocks the phosphorolysis, just as it blocks further addition. The presence of a 5'-phosphate is not essential and not inhibitory for either hydrolysis or further synthesis. The presence of 2', 3'-cyclic phosphate also blocks the action of the

enzyme. The blocking action of the 3' phosphate or cyclic phosphate is in line with the finding that splitting begins at the 3' end, just as further addition is here. The enzyme will act on a trinucleotide (pApApA) or a trinucleoside diphosphate (ApApA) but will not act on a dinucleotide (pApA) or a dinucleoside monophosphate (ApA). A tetranucleotide is split more rapidly than a trinucleotide. Ochoa[44] has shown that the higher polymers such as poly A or poly U are readily phosphorolyzed when present alone but in combination they form multistranded chains and these are resistant to the enzyme.

B. *Studies with homogenates and cell fractions*

After finding the 5'-ribonucleotides of all the RNA components in the acid soluble fraction[4] our next studies were directed toward the enzymes for converting the monophosphates to the di- and triphosphates (Chapter IX), and to the conversion of the latter to polynucleotide form. Experiments in collaboration with Hecht and Herbert[33, 50] established systems for converting C^{14} from orotic acid into the uridine 5'-nucleotides and into RNA.

In these first reports of incorporation of labeled nucleotides into the RNA of cell-free systems from mammalian tissue it was important to show that the results did not depend on the use of homogenates, which might contain whole cells, and to establish that labeling of RNA depended on the presence of uridine-5'-phosphates. It was emphasized that in these systems there was no formation of labeled cytidine 5'-phosphates which was correlated with the fact that there was no labeled cytidylic acid in the RNA[33, 50]. It was later shown by Kammen and Hurlbert that the conversion of uridine 5'-phosphates to the cytidine 5'-phosphates requires the presence of glutamine (Chapter VI).

It was established that both the microsome frac-
tion and the soluble fraction were necessary for
maximum RNA labeling, while the mitochondria
were unnecessary except as oxidative generators
of ATP[33].   The chief value of these studies was to
demonstrate incorporation of labeled nucleotides
into RNA in a cell-free system.   Although labeling
occurred in nuclear fractions little emphasis could
be placed on the data because such preparations
always contain some whole cells.   However a use-
ful preparation is the supernatant fraction from the
nuclei of homogenates, prepared by centrifuging
for ten minutes at 600 x g.   This fraction contains
the mitochondria, the microsome fraction, and the
soluble supernatant or S_3 fraction, and is called
the "cytoplasmic fraction" or S_1.   A supernatant
fraction obtained after 10 minutes at 500 x g eli-
minates the mitochondria and is called an S_2 frac-
tion (Chapter IV).   Both types of preparations were
used.

In order to establish that the uridine 5'-phosphate
had been incorporated into RNA, we made use of
the fact that alkaline hydrolysis of RNA leads to
production of uridine 2'(3')-phosphate (Chapter II)
while the acid soluble fraction contains only the 5'-
phosphates, plus the fact that the 2'(3')-phosphates
can be readily separated from uridine 5'-phos-
phate on ion exchange columns.   When RNA was
extracted from the acid-insoluble material follow-
ing protein precipitation, and hydrolyzed and
chromatographed, uridine 2'(3')-phosphate was
recovered, showing that it had been in material
having the properties of RNA.   Since the 2'(3')
phosphate of uridine was recovered it appeared
that the incorporation was followed by the incor-
poration of other nucleotides with sufficient fre-
quency to place the majority of the labeled uridylic
moieties at least one nucleotide away from the 3'
end which is considered to be non-phosphorylated
by all available evidence.   The presence of small

amounts of ATP, less than 5×10^{-4} M, was stimu-latory to the overall system incorporating labeled orotic acid into RNA in the form of uridylic acid by virtue of increased conversion of orotic acid into uridine nucleotides. When the ATP concentra-tion exceeded this amount it decreased the incor-poration of uridine phosphate into RNA, probably by competition between uridine phosphates and adenosine phosphates. That adenosine phosphates could be incorporated into RNA in this system was shown in collaboration with Takagi and Heidel-berger, Leibman and Harbers[31]. Adenosine 5'-phosphate labeled with P^{32} was incorporated into RNA but gave the surprising result that on alkaline hydrolysis nearly all of the radioactive P^{32} was recovered in 2'(3') cytidylic acid. That the P^{32} was still attached to the adenosine moiety was proved by hydrolyzing with snake venom diester-ase, in which case nearly all the P^{32} was recover-ed as adenosine 5'-phosphate. These experiments proved that the labeled AMP was entering a poly-ribonucleotide in a linkage of the following type:

$$- - - - pCp^{32}A - - - -$$
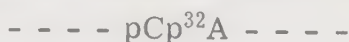
and the most likely explanation would be that the $P^{32}A$ moieties preferentially entered RNA termi-nated on the 3' end by a cytidine, i.e., RNApC. This finding was confirmed by Canellakis[14], and was later extended in elegant fashion by Hecht, et al.[28, 30] and Hoagland et al.[36] who established the preferential sequential labeling of the end groups of soluble RNA to give RNApCpCpA which could serve as a mechanism for amino acid trans-fer. The Zamecnik group[28, 30, 36] also showed that the actual precursors were the ribonucleoside tri-phosphates and not the diphosphates as might be in-ferred from the studies with the Ochoa enzyme[22, 23]. Our earlier studies had not established whether di-phosphates or triphosphates were the actual pre-cursors because both were present and rapidly interconverted in these systems.

The studies on soluble RNA[28, 30, 36] show that a large proportion of the incorporated adenosine 5-phosphate is on the 3' end of the RNA since it yields adenosine upon alkaline hydrolysis. This result was also observed by Paterson and LePage[48], however in a considerably different type of system. Other studies with labeled adenine nucleotides[19, 20, 32] and cytidine nucleotides[66] have shown non-terminal labeling. If the labeling of RNA in these studies is a result of incorporation of nucleotides one at a time on the 3' end as in the case of the Ochoa enzyme system, the question of non-terminal or terminal addition may be merely a question of the total time elapsed and of the number of available end groups in relation to the supply and variety of precursors. Schneider and Potter[59] worked with a system in which labeled uridine-5'-phosphate moved into the microsome fraction. In this system the incorporation was accompanied by loss of label from the RNA and it was shown that the longer the incorporation had proceeded, the less likely was the label to be lost from the RNA, as if the incorporation was at the 3' end and became "protected" by further incorporation of non-labeled nucleotides with time.

The data on terminal labeling in soluble RNA by nucleoside triphosphates and the lack of clear-cut demonstrations of incorporation of nucleoside diphosphates has led to an impression that the Ochoa system does not operate in mammalian tissues, but this conclusion would be premature at this time. The next step is to attempt to obtain net synthesis of RNA with purified enzyme systems from various cell fractions, and to distinguish between the RNA that is assumed to be needed for template function in protein synthesis, and the RNA that is needed for amino acid transfer. At this time it is not clear whether template RNA is always made directly from a DNA or DNA-protein template or whether some template RNA is self-

duplicating in the presence of the proper enzymes and substrates. It seems probable that both types of synthesis must occur and that the demonstration of either one should not be considered proof of the non-existence of the other. Since the labeling of soluble RNA has surely complicated most previous studies on RNA synthesis, it seems worthwhile to learn as much as possible about the properties of this system, in order to be able to either exclude it from studies on template RNA or to learn whether it is involved in the synthesis of the latter.

C. *Soluble RNA*

The first full report on soluble RNA was published by Hoagland et al. in 1958[36]. This paper established the existence of a relationship between protein synthesis and the RNA that occurs in the S_3 fraction of cytoplasm from rat liver or other cells. This RNA remains in solution when the microsome fraction and most of the ribosomes are sedimented at 105,000 x g for 90 to 120 minutes, and is precipitated when the pH is lowered to 5.0 or below. In subsequent reports from this group, Hecht et al.[29, 30] established many of the salient features of the soluble RNA system, and these two papers should be read in their entirety. It was shown (Table I[30]) that the incorporation of each of the 4 ribonucleoside triphosphates was affected differently by the presence of the other 3, and preferential incorporation was demonstrated: Labeled CTP was incorporated to a maximal level whether alone or in the presence of ATP, UTP, or GTP taken singly or in any combination. Labeled ATP was not incorporated maximally when added alone, but was brought to the maximum level in the presence of CTP, and this level was not altered by GTP or UTP singly or together. UTP was poorly incorporated alone, and its incorporation was further reduced in the presence of CTP or

GTP. GTP was very poorly incorporated alone or in various combinations. These data were not in line with the idea of net synthesis of RNA, in which the incorporation of single nucleotides should be very low and should be greatly enhanced by the presence of the other 3 building blocks as in DNA biosynthesis or in certain types of RNA labeling[66]. Instead they supported the idea of a preferential terminal sequence of nucleotides, and strongly supported the previous suggestion[31] of a RNApCpA sequence. (Note: in this report[30] the notations are all written in the reverse direction compared to the currently accepted convention). If this formulation were correct, and a particular type of RNA could be terminally labeled in this manner, one might expect that native soluble RNA would be a mixture of the following types:

$$RNA \underset{\longleftarrow}{\overset{CTP}{\longrightarrow}} RNApC \underset{\longleftarrow}{\overset{ATP}{\longrightarrow}} RNApCpA$$
preincubation
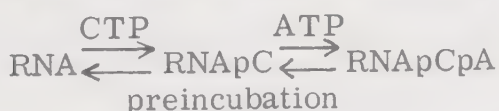
and that incubation in the presence of the S_3 enzymes in the absence of CTP, ATP, UTP and GTP would result in formation of soluble RNA lacking pC or pCpA. The authors accordingly carried out "pre-incubation" experiments to remove the pCpA end groups and then carried out the addition incubations in the presence of CTP or ATP. Pre-incubation reduced the ability of RNA to accept pA from ATP but in the presence of CTP the ability to incorporate pA was restored, again indicating that pA could add to RNApC but not to RNA not terminated by pC.

Further studies on the location of the additive nucleotides in the labeled RNA molecule was obtained by alkaline hydrolysis of RNA labeled under various conditions (Table IV[30]). In all cases of incubation of labeled A*TP the pA* moiety was split off the labeled RNA as adenosine (85-90%) according to the proposed labeling RNApCpA*. In contrast,

the position of C with respect to the 3' end depend-
ed on whether ATP was present. When C*TP
alone was added it was obtained in almost equal
proportions of Cp and C, and this result was not
affected by the presence of GTP or UTP. But
when ATP was added the labeled C* appeared to
be covered by pA because after alkaline hydroly-
sis it was recovered as 89% C*p:

RNA + C*TP → RNApC*pC*

RNApC*pC* + alkali → RNAp/C*p/C* → 51%C*p + 41% C*

RNA + C*TP + A*TP → RNApC*pC*pA*

RNApC*pC*pC*A* + alkali → RNAp/C*p/C*p/A* → 89%C*p + 90%A*

These experiments suggested that there were ac-
tually two Cp units in the final product, arranged
as shown above, and measurements of the absolute
amounts of C incorporated revealed that this fig-
ure was about 2x as great as the amount of A in-
corporated. It appears likely that the same data
could be obtained if some of the pCpC groupings
were separated by one or more other nucleotides.
Evidence that the linkage in the terminal CpC unit
is more labile than the others was indicated by the
isolation of labeled CpA by mild alkaline hydroly-
sis of RNA labeled in the presence of both CTP
and ATP[30].

In the second paper recommended for reading[29] the
above experiments were carried out in terms of
the incorporation of labeled amino acids into solu-
ble RNA according to the techniques previously
developed.[36] Preincubated RNA is unable to accept
activated amino acids unless both CTP and ATP
are present, but RNA previously labeled with CTP
is able to incorporate amino acids (aa) with only
ATP present.   These studies led to the conclusion

that soluble RNA can occur in all of the following forms:

RNA, RNApC, RNApCpC, RNApCpCpA, and RNApCpCpA-aa

It was shown that each of the forms shown is capable of being converted to either the compound listed ahead of it, or to the compound listed after it, depending on the presence or absence of CTP, ATP and activated amino acids. The incorporation of the pC and pA units is a reversible reaction in which inorganic pyrophosphate is one of the reactants, for example

$$RNApCpC + ATP \rightleftharpoons RNApCpCpA + pp$$

In experiments with mixtures of amino acids, the total incorporation was shown to be equal to the sum of the individual maximum levels of incorporation. This was explained by assuming that a different kind of an RNApCpCpA is needed for each amino acid, and subsequent work by Smith, Cordes and Schweet[64] has pointed the way toward further experiments to test this idea by achieving a partial separation of the "soluble RNA that accepts tyrosine" from the "soluble RNA that accepts leucine"; the latter was prepared nearly free of the tyrosine-accepting type of RNA. A cationic starch exchanger was eluted with NaCl and the eluates were tested for amino acid acceptance after separation.

Work by Hecht et al.[29], by Zachau et al.[67], and by Preiss et al.[51] led to the firmly established conclusion that the amino acids that are attached to soluble RNA are bound through an acyl linkage to either the 2' or the 3'OH of the terminal adenosine. In the compound RNApCpCpA-aa treatment with ribonuclease splits the phosphate diesters in the usual manner without splitting the adenosine-amino acid ester, giving Cp and A-aa.

The naturally occurring soluble RNA is apparently
saturated with amino acids,[1] in a linkage which is
extremely labile to alkali[36] or to enzymatic cleav-
age in the preincubation procedure of Hecht et al.[29,30]

This section should not be closed without mention
of the growing literature on the presence of high
proportions of pseudouridine[2, 15] in soluble RNA[17, 18]
as contrasted to amounts in other types of RNA.
The possible role of this compound in amino acid
transfer is being studied by Osawa et al.[47]

At present the assignment of specific roles for
soluble RNA that contains pseudouridine (Chapter
II) must be postponed until it can be learned wheth-
er the compound is present in the soluble RNA
corresponding to all of the amino acids or only in
RNA's that are specific for certain amino acids.
The occurrence of separate RNA molecules for
each type of amino acid is now widely assumed but
none have been prepared in sufficient quantity to
permit determination of base ratios, including the
amounts of the exotic components.[18] The assumed
coding of the amino acid transfer specificity by
specific sequences of bases (Chapter X) may or
may not turn out to involve the exotic components
but in any case it is not at all understood at pres-
ent because of the redundancy presented by the
fact that soluble RNA has an average molecular
weight of about 30,000 and contains 80 to 90 nucleo-
tides per molecule. It seems conceivable that
shorter segments of soluble RNA could accept
activated amino acids but be unable to transfer
them to polypeptide linkage in the ribosomes.

D. *Ribosomal RNA*

At the present time, there is excellent data to
show that amino acids bound to soluble RNA in
non-peptide linkage are transferred to ribosomes,

where they go into peptide linkage, forming pro-
tein[35]. For example a reciprocal relationship
showing loss of labeled amino acid from soluble
RNA and increase in the label in microsomal pro-
tein was shown by Hoagland et al (Fig. 6)[36]. How-
ever, similar data are not as available in the case
of the labeled nucleotides in soluble RNA. It
seems likely that the soluble RNA should occupy
sites on ribosomal RNA while unloading amino
acids, but the question at this time is whether the
nucleotides in ribosomal RNA enter by way of solu-
ble RNA or by some other pathway. This question
has been taken up in E. coli by the group in the
Biophysics section of the Carnegie Institution of
Washington in connection with their studies of ribo-
some function[13, 53, 54, 55]. They separated soluble
RNA from ribosomes by sedimentation, column
chromatography and electrophoresis and concluded
that the soluble RNA is not a precursor of ribo-
somal RNA. It appeared to be a relatively stable
end product of synthesis since its P^{32} content was
undiminished after several generations growth in
P^{31} medium. They observed no exchange of phos-
phorus between soluble RNA and ribosomal RNA.
They found that the ribosome content varies from
50 to 85% of the cell RNA being highest during
rapid growth. After prolonged growth in a synthe-
tic medium that contained P^{32} and permitted a slow
rate of growth, the specific activities of the solu-
ble RNA and the ribosomal RNA were the same.
On transferring the cells to a P^{31} medium that in
addition permitted an increased rate of growth,
there was an increase in the ratio of ribosomal
RNA to soluble RNA. The specific activity of the
ribosomal RNA fell relative to that of the soluble
RNA, which was taken to mean that newly synthe-
sized ribosomal RNA drew nucleotides from the
unlabeled pool with no exchange with the soluble
RNA.

Studies on the ribosomes showed that a variety of sizes were present in the E. coli cells during growth, ranging from those having sedimentation constants of 80 to 100s down to values of 70s, 50s, 30s, 20s. Molecular weights for all of these classes are not available but Hoagland[35] summarizes some of the available data[53, 65]. The 70s particles from E. coli have a molecular weight of about 2.8×10^6 and are about 60% RNA and 40% protein. The 80s particles from yeast and from liver have been given as about 40% RNA and 60% protein, with a molecular weight of about 4.1×10^6. At concentrations of Mg ion around 10^{-2}M the 80 to 100s particles predominate and the 50s and smaller particles are at their lowest concentration. As the Mg concentration is lowered the 80-100s class seems to be converted to 70s particles and further reduction of the Mg concentration toward 10^{-3}M causes the 70s particles to dissociate into equal numbers of 50s and 30s particles.[13, 65] The latter are considered to be separate entitities in the sense that the 50s particle is not a simple combination of 2 of the 30s particles, but 30s particles may pick up nucleotides and amino acids and grow to form 50s particles, while similarly the 20s particles could grow to form 30s particles. Since the particles in living cells appear to undergo all of these changes it is very difficult to decide precisely which ribosomal particles represent sites of RNA synthesis and which represent products of prior synthesis in a different particle. Thus it was found that P^{32} flowed from the smaller particles to the larger ones, but the reverse flow could also be shown. The present view seems to be that the synthesis of the ribosomal RNA is partly autonomous and partly dependent upon DNA, as if there were a production of new RNA from DNA templates, while the new RNA could grow into ribosomal RNA and reproduce independently of DNA within the limits of its survival time. The

present models of template RNA synthesis are
compatible with the idea that the 20s particles may
come from the nucleus and provide the starting
point for further autocatalytic ribosomal RNA syn-
thesis in which 20s particles could also be regen-
erated from 30s particles and 30s particles could
be regenerated from 50s particles. Figure 7.2 is
an attempt to diagram data such as that obtained by
the Carnegie group[13].
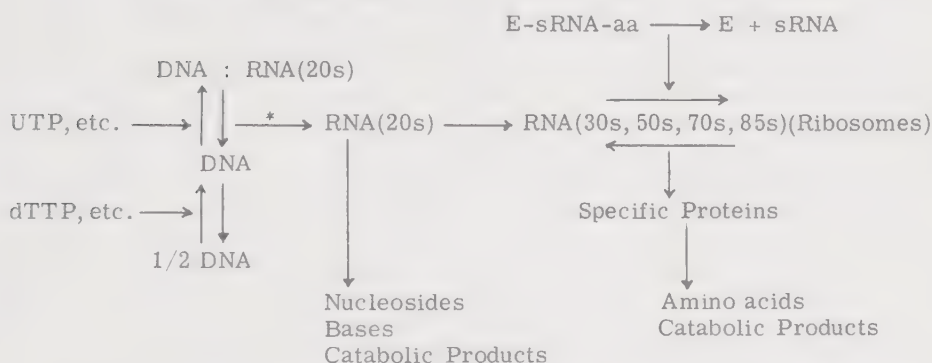
Fig. 7.2. Possible relationships between DNA and ribosomal RNA classes, arranged to
facilitate discussion of isotope incorporation data. (Modified from Fig. 40[13]
to include RNA decay and a role for DNA). In the diagram sRNA = soluble
RNA, aa = amino acids, and 20s, 30s, etc. refer to the various sedimenta-
tion classes of ribosomal particles. The significance of the asterisk is dis-
cussed in the text as a step that may be radiation sensitive.

The relationships shown in Fig. 7.2 are based in
part on the findings with magnesium-starved E.
coli after magnesium has been restored to the
medium[13]. Magnesium starvation reduced the ribo-
some content to as little as 5% of normal, with ri-
bosomal RNA passing into acid soluble forms
irreversibly. Recovery proceeded by de novo syn-
thesis of ribosomal RNA, which increased expo-
nentially by a factor of more than 80. Since the
increase seemed to be autocatalytic it was inferred
that it was a "reasonably autonomous" system,
hence the right-hand portion of Fig. 7.2. Con-
versely, a thymine-requiring mutant of E. coli,
15 T⁻, was able to synthesize RNA but not DNA
when thymine was depleted, but the rate of ribo-

some synthesis stopped increasing when the <u>amount</u> of DNA stopped increasing, hence the left-hand portion of Fig. 7.2. It remains to be seen whether comparable experiments can be carried out in animal tissues: it may be possible to block the hypothetical production of 20s particles by DNA (see asterisk in Fig. 7.2) by X-radiation since we have described the prevention of certain enzyme syntheses in regenerating rat liver[9] by as little as 375 roentgens given prior to the appearance of enzyme forming units. In addition, the autocatalytic functions of the 20s particles might be greatly damped in rat liver by the use of low protein diets.

It is clear that the experimental techniques for testing schemes such as shown in Fig. 7.2 are still in their infancy. Although much can be done with soluble enzymes there is an even greater task to be carried out with the particulate units shown in Fig. 7.2. At the moment the production of specific proteins in cell-free systems seems to be experimentally attainable (Chapter XII) while the production of specific RNA seems more difficult. However if it turns out that the generation of specific protein can serve as the test for the generation of specific RNA, appropriate cell-free systems may be developed.

III.   BIOSYNTHESIS OF DEOXYRIBONUCLEIC ACID

A.   The role of primer

The first successful system for the synthesis of DNA established the need for the presence of preformed DNA in the reaction mixture, and this preformed DNA was referred to as "primer"[39b]. A subsequent study[39g] set out to answer the questions: (1) Does the synthesized DNA show the equivalence of purines to pyrimidines that is characteristic of DNA and in agreement with the Watson-Crick hypo-

thesis of A equal to T and G equal to C? and (2)
Does the base composition of the DNA primer in-
fluence the composition of the synthesized product?
The results answered both questions in the affirm-
ative, suggesting that "from the very outset of the
reaction" the added DNA "is serving as a template
for the synthesis of new DNA". The authors were
able to obtain DNA from a number of sources that
yielded ratios of $\frac{A + T}{G + C}$ ranging from 0.49 (Myco-
bacterium phlei) to 1.92 (T_2 bacteriophage) and
when these were used as primers, the correspond-
ing ratios in the products were reasonably close
to that of the primer, although some of the devia-
tions could be significant. The most dramatic
result was obtained when a "DNA" containing only
A and T was used as primer. This A-T polymer,
incidentally follows the Watson-Crick pairing in
that the ratio of A to T is almost exactly 1.0
(1.05), and it is inferred that it is double-stranded,
with A and T alternating in each. When the A-T
polymer was used as primer, the product contain-
ed only deoxyadenylic and thymidylic acids, even
in the presence of all four deoxyribonucleoside
triphosphates in the reaction mixture. This ex-
periment was widely acclaimed as one of the
strongest supports for the Watson-Crick hypothesis
to date. The source of the primer was of interest,
since no previously reported DNA had been found
free of G and C. It was reported that the A-T
polymer was formed in the absence of added pri-
mer after a lag of 3-6 hours. Once formed and
added to a new reaction mixture, more A-T poly-
mer was synthesized with no time lag. A possible
explanation might be that the polymerase can form
A-T polymers from the mononucleotides at a very
slow rate.

Experiments with animal tissues[10],[11] also showed
an absolute requirement for DNA primer in the
action of DNA polymerase, and proved to be very

useful for demonstrating a further requirement in the nature of the primer: in a paper entitled "Thermal Conversion of Non-priming Deoxyribonucleic Acid to Primer" Bollum[6] showed that highly purified preparations of DNA were relatively ineffective as primers unless they were heated, and proposed that only single-stranded DNA can serve effectively as primer. It appears that the Bollum preparations of DNA polymerase from calf thymus have been nearly free of naturally-occurring enzymes that must be involved in converting non-priming DNA to priming DNA when DNA synthesis is initiated in living cells, while the preparations from E. coli must have contained such enzymes in the early phases of the work. Whether such enzymes are related to the several types of DNAses reported[39c] in even the best preparations of bacterial DNA polymerase remains to be seen. In the following discussions of DNA polymerase it will be understood that primer DNA was always present.

B.   DNA polymerase from bacteria

The properties of the bacterial DNA polymerase system were described in 1958 in a classic series[39] of papers (I-V) from Kornberg's laboratory. Paper I describes the preparation of the 4 labeled deoxyribonucleoside triphosphates and preparation of the enzyme from E. coli. Paper II describes the general properties of the reaction, which consists of the incorporation of the deoxyribonucleoside monophosphate moieties of the deoxyribonucleoside triphosphates of thymine, adenine, guanine and cytosine into polydeoxyribonucleotide form with one mole of inorganic pyrophosphate liberated for each mole of triphosphate that enters the reaction. Mg ion is required. The net synthesis of DNA, i.e. detectable increase in DNA above the amount used as primer, could be shown

to yield amounts of DNA 2 to 20 x greater than the starting amount. In these reactions, the omission of any one of the necessary deoxyribonucleoside triphosphates made the reaction fall to about 0.5% of the maximal value. The latter reaction was called "the limited reaction" and it might be attributed to the inability of the system to bridge sequences of nucleotides where no base pairing was possible. That is, if each mononucleotide has to be added to a growing strand at a point opposite its Watson-Crick partner according to the A:T, G:C rule, one might expect that if only one type of triphosphate were available it might go into those strands where pairing was possible but that the chain could not be extended because of a lack of the next nucleotide appropriate for the sequence.

1. The "limited reaction". - The authors found that in a limited reaction with dCTP this nucleotide alone was incorporated to the extent of only 2.5 $\mu\mu$ moles as measured by P^{32} incorporation. When dGTP was added in addition the number was 5.1, and even with added dGTP and dTTP the number was only 15.7 as compared to 3300 when dCTP, dGTP, dTTP and dATP were all present. This type of reaction was studied further in Paper IV[39f] and the extent of the "limited reaction" with dATP, dCTP, or dTTP alone (dGTP not reported) varied from 0.13 to 0.34 mμ moles per milliliter of reaction mixture when taken one at a time, corresponding to approximately one mole of precursor per mole of DNA. It was shown that the entering nucleotide attached its 5'-phosphate to a free 3'OH presumably at the end of a sequence of polymerized nucleotides. However the authors did not conclude whether it added to the end of a single strand DNA, to the shorter of two unequal paired strands, or whether it formed hydrogen bonds to corre-

sponding Watson-Crick nucleotides in the
primer chain followed by elimination of pyro-
phosphate (presumably wherever 2 or more
repeats of the given nucleotide could be align-
ed).   Perhaps a fourth possibility is that the
incorporation is a measure of the extent of
the reaction with the 3' OH of the terminal
nucleotides (one on each end of a double helix)
when there is no hydrogen bonding at all.
However it appears that the data support the
explanation involving paired strands of unequal
length, as will be emphasized in the discus-
sion to follow.   When the authors used C^{14}
labeled dCTP 67% of incorporated nucleotide
was recovered as C*dr and 33% as d-C*p
(Table 2)[39f] when the DNA was hydrolyzed by
a diesterase with action like that of alkali on
RNA (Chapter III).   This result could be ex-
plained by assuming that the product was 50%
DNA-p/Xp/C* and 50% DNA-p/Xp/C*p/C*
and that little if any DNA-p/Xp/C*p/C*p/C*
was formed.   (Bars indicate diesterase action
and X = C, G, A or T).   When d-ppp*C (p* = P^{32})
was used as precursor, 34% of the radioactiv-
ity was recovered as Cp*, a result that can
be interpreted in the same quantitative terms
as the C^{14} data.   Since the reaction had a
rapid initial reaction and a much slower reac-
tion between 30 and 120 minutes, it would be
of interest to learn whether at 10 or 20 min-
utes the product would have been predominant-
ly DNA-pXpC* (or DNA-pXp*C if P^{32} was used)
and predominantly DNA-pXpC*pC* at longer
time periods.   In experiments with dppp*A or
dppp*T the hydrolysates yielded 57% Ap* and
52% Tp* respectively while the Xp* values
averaged 14% and 16% respectively.   These
results can be explained if we assume that the
reaction had gone essentially to completion,
in which "completion" is extension from the

3'OH end of the shorter member of a DNA
double strand to 2 additional nucleotides, and
it is assumed that the first addition is rapid,
the second addition is slow, and a third addi-
tion insignificant. In the "limit" products
formed by adding dppp*A to a DNA primer we
would accordingly expect to find
DNA-p/Xp*/Ap*/A. If in the original DNA
primer there was an equal probability that X
would be A, T, C, or G, the four types of
labeled DNA molecules could be represented
as follows, omitting the hydrogen bonding to
the second strand, to which none of the label-
ed precursors attach by covalent bonds:

> DNA-p/Ap*/Ap*/A
> DNA-p/Tp*/Ap*/A
> DNA-p/Cp*/Ap*/A
> DNA#p/Gp*/Ap*/A

Hydrolysis as indicated by the bars would
yield labeled products 62.5% Ap* and 12.5%
of each of Tp*, Cp*, and Gp*. Correspond-
ing percentages would be obtained if T were
substituted for A in all cases. These numbers
are actually quite close to the actual data cited
above. With the single precursor ppp*A the
products were 57% Ap*, 15% Tp*, 6% Cp*,
and 22% Gp*. With the single precursor
ppp*T the products were 52% Tp*, 17% Ap*,
10% Cp*, and 21% Gp*.

These results clearly establish the fact that
the "limited" reaction involves the formation
of covalent bonds and in addition strongly sug-
gest that hydrogen bonding is necessary for
additional lengthening of the chain. The re-
sults can best be explained by assuming that
the addition of single nucleotides "is merely
part of the extension of the shorter of two un-
equally long chains of a DNA double helix",

one of the possibilities considered. It is
merely necessary to assume (1) that the first
nucleotide to be added is covalently bonded to
the 3'OH end of the shorter chain at a point
that permits it to be hydrogen bonded to its
appropriate nucleotide in the opposite strand
according to the A:T, G:C rule, and (2) that
one and only one additional nucleotide can be
added if the base in the pairing position is not
capable of hydrogen bonding. This relation-
ship is shown in Fig. 7.3.

```
              etc.
           p-dr-G
           /
           p-dr-C
           /                        /
           p-dr-A           p*-dr-A
           /                      /
           p-dr-T...A-dr-p*
           /                      /
           p-dr-X'..X-dr-p
           /                      /
           p-dr-X'..X-dr-p
           /                      /
           p-dr-X'..X-dr-p
```
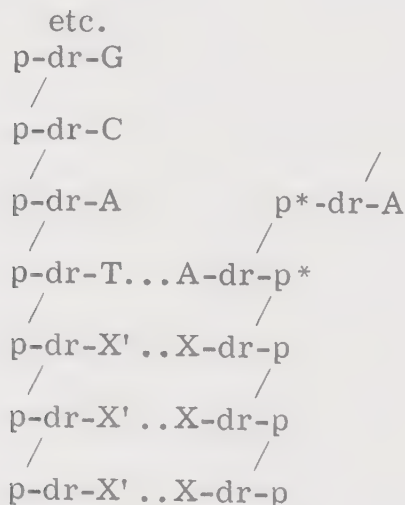
Fig. 7.3. "Limited" incorporation of dppp*A as the sole available
          precursor into DNA primer assuming double strands of
          unequal lengths in order to explain labeling of hydroly-
          sis products[39f] and extent of labeling with other precur-
          sors added 1, 2, or 3 at a time[39d] as described in text.
          Notation as in Fig. 7.1 with X= A, C, T, or G, and
          X' = Watson-Crick pairing base. The limited reaction
          occurs in two stages: the first A-dr-p* goes in opposite
          a T in the paired strand, while the second A-dr-p* is
          shown unpaired and rotated to an extreme position to
          emphasize lack of hydrogen bonding.

It appears reasonable to assume that the
second or non-hydrogen bonded nucleotide in

this figure would be more subject to removal by pyrophosphorolysis than the first nucleotide. It appears that the "limited reaction" may be separated into a one-stage limited reaction, and a two-stage limited reaction, and the above interpretation infers that in double stranded DNA with matching lengths, only the second stage of the limited reaction would be possible in the absence of factors converting non-primer to primer. Experiments with single strand DNA might be difficult to interpret because of possible random coiling, with hydrogen bonding of a strand folded back upon itself. Further discussion of primer function will be given in the section on mammalian DNA polymerase.

2.   Incorporation of pyrimidine and purine analogs.[39e] - In Paper III of the Kornberg series it was shown that a number of purine and pyrimidine analogs could be incorporated into DNA if presented in the form of the deoxyribonucleoside triphosphates. The latter point needs to be emphasized because it was shown for example that dUTP was readily incorporated, yet no uracil has been reported to be in naturally occurring DNA. This was explained by reference to the fact that no dUMP kinase has yet been discovered, hence in cells there appears to be no conversion of dUMP to either dUDP or dUTP. The authors prepared the deoxyriboside triphosphates of uracil and 5-bromouracil and found they would replace TTP; of 5-methyl-cytosine and 5-bromocytosine and found they would replace dCTP; of hypoxanthine and found it would replace dGTP; of xanthine and found it would not replace dGTP. In all cases of incorporation the results were compatible with the Watson-Crick base-pairing rules, while the result with dXTP showed that compatible hydrogen bonding was no

guarantee that a compound would be incorporated. These results assume considerable significance as background for the studies on the mutagenic effects of various purine and pyrimidine analogs as carried out by E. Freese (Chapter X) in which it was shown that certain mutations occur in much greater frequency than others when specific analogs are used: the analogs, by replacing specific natural bases might be expected to produce mutations in something other than a completely random manner.

C. *DNA polymerase from mammalian tissues*

The first successful experiments on DNA-polymerase from animal tissues were carried out in 1957 by Bollum and Potter[10] using homogenates of regenerating rat liver. It was shown that the soluble supernatant fraction from the homogenates contained not only DNA polymerase but also thymidine kinase, thymidylic kinase, and the kinases for dAMP, dGMP, and dCMP, since in the presence of these three monophosphates, ATP, and primer DNA it was possible to obtain incorporation of tritium-labeled thymidine into DNA[12]. An absolute requirement for primer DNA was shown[11] and maximal incorporation of labeled TTP was dependent upon the presence of all three of the other deoxyriboside triphosphates[5]. Assays were developed on the basis of the over-all reaction from thymidine to DNA (called kinase-dependent) or on the basis of the incorporation of preformed triphosphates (the kinase-independent assay)[6]. In the absence of primer DNA, thymidine or TMP can still be converted to TTP in the presence of ATP.

With the above facts in hand it was possible to follow the appearance of the kinases and the polymerase as a function of time after partial hepatec-

tomy in the rat. These studies were an extension
of earlier work by Hecht and Potter who studied
labeling of DNA in whole animals[25, 26] and in
slices[27]. This work established a close correla-
tion between labeling of DNA in vivo and in slices,
and together with the work of Beltz, Van Lancker
and Potter[4] established that not only the labeling
but the absolute increase in DNA began approxi-
mately 18 hours after partial hepatectomy. Bol-
lum and Potter[12] showed that these events also
coincided with a marked increase in the amount of
the kinases and the DNA polymerase in the soluble
cytoplasmic fraction. Hiatt and Bojarski[34] con-
firmed the time relationships using an assay
specific for TMP kinase and in addition showed
that the amount of this enzyme could be markedly
increased over a period of 5 hours in normal rat
liver (i.e. without partial hepatectomy) by injec-
ting large amounts of thymidine (100mg) at hourly
intervals.

On the basis of the fact that the enzymes are in the
soluble cytoplasmic fraction, Bollum and Potter[12]
were able to correlate the enzyme activity in vitro
and the DNA synthesis in vivo in the same individ-
ual rats. The animals were injected with labeled
orotic acid at various times after partial hepatec-
tomy and killed two hours after the injection.
Homogenates were prepared and the nuclei were
centrifuged down and used for the determination
of DNA labeling in vivo. The soluble supernatant
fraction was then used for the kinase dependent
assay with labeled thymidine, ATP, and unlabeled
primer DNA. There was a good correlation be-
tween the DNA labeling in vivo and the in vitro in-
corporation of thymidine into primer DNA in the
period from 0 to 24 hours post-hepatectomy, with
a steep rise beginning at 18 hours. After about 24
hours the in vivo activity diminished markedly al-
though the amount of activity shown by the enzyme
assays continued to rise for another 24 hours.

This was interpreted to mean that in vivo only a limited number of cells synthesize DNA at any one time, while their cytoplasm retains the DNA-synthesizing enzymes for some time after endogenous primer DNA is no longer available.

The occurrence of DNA polymerase in animal tissues was confirmed by Mantsavinos and Canellakis[42] and by Davidson et al[16].

1. DNA polymerase from calf thymus. Using calf thymus as a source material Bollum[6] has been able to carry out a number of extremely important studies on DNA polymerase. The thermal conversion of native "non-priming" DNA to priming DNA added much support to the concept that single strand DNA is the form required for replication. Further advances have been made possible by using synthetic primers generously provided by Dr. H. G. Khorana. These primers consisted of dpTpTpT and several higher homologues. The trinucleotide is shown in Fig. 7.1, I and II, which possibly illustrates the two types of results that have been obtained[8]. The trithymidylate served as primer using dATP as substrate and the product yielded a single spot on a paper chromatogram, possibly with the structure shown as a product in Model II, and in view of results obtained with other single triphosphates as substrates. When dCTP was used, the results were similar to those obtained with dATP. When dGTP was used, not one but a whole series of discrete products appeared as spots on the paper chromatogram, and similar results were obtained with dTTP. If the latter two compounds added to the 3'OH of trithymidylic primer as shown for dGTP in I a series of products containing the primer plus 1, 2, 3, 4 or more dGMP (or dTMP) moieties might be found.

The results with small primers suggest how the lag period and production of poly AT without primer may have taken place in the experiments of the Kornberg group[39g] and seem to open up some interesting possibilities for the further understanding of primer function.

IV. INCORPORATION OF RIBONUCLEOTIDE INTO DNA

An interesting development has been reported by Hurwitz[38a], who was able to incorporate a labeled ribonucleotide into DNA, using ppp*C as substrate and an enzyme prepared from E. Coli. It was established that in the presence of DNA primer, dTTP, dATP and dGTP, CTP could be incorporated into a polynucleotide form in which pC appeared to be interspersed among deoxyribotides in such a way that the 5'-phosphate became the 3' phosphate of the adjacent deoxyribonucleotide when the procedure of Adler et al[39f] was used to hydrolyze the polymer to the 3'-phosphate mononucleotides. The data appear to be explainable along the same lines we used to explain the "limited reaction" described by the Kornberg group (Fig. 7.3), followed by continuing incorporation of CMP by extension of the shorter member of a two-stranded primer in in the presence of dATP, dGTP and dTTP. Indeed, one wonders how such incorporation could be avoided in a cell free system containing the proper enzymes and substrates, and at this writing, there is a real possibility that the results may be due to an artifact since it seems possible that the results could be brought about by a mixture of DNA polymerase and an RNA polymerase, either of the Ochoa variety acting on ribosidediphosphates or of the type described by Hecht et al[30] which acts on triphosphates. If not an artifact the synthesis of a mixed polymer would be an important discovery much in need of further explanation.

V. THE INTRACELLULAR LOCALIZATION OF NUCLEIC ACID SYNTHESIS

The above studies taken in toto add very little to the problem of the intracellular localization of nucleic acid synthesis. The isolation of soluble enzymes from either nuclear or cytoplasmic fractions is no guarantee that the enzymes did not originate in another part of the cell and such data must be supported by collateral data obtained with intact cells. Studies with intact and enucleated amoebae have led to sharply contrasting conclusions: Plaut and Rustad[49] concluded that the nucleus played a relatively unimportant role in the synthesis of cytoplasmic nucleic acid while Prescott[52] concluded that RNA synthesis is completely dependent upon the nucleus. Zalokar[68] working with Neurospora crassa obtained data favoring the nuclear origin of RNA, while Harris[24], working with animal cells in tissue culture obtained results that were considered incompatible with the view that cytoplasmic RNA comes from the nucleus.

A somewhat different approach to the problem was described by Hecht and Potter[27] who reported on studies using slices of regenerating rat liver incubated in the presence of labeled orotic acid. After various periods of time aliquots of slices were chilled, homogenized and centrifuged to obtain nuclei, soluble supernatant fraction, microsomal fraction and mitochondrial fraction. The results were quite striking in that a linear incorporation of C^{14} into RNA was demonstrated for the slices as a whole for the 4 hours of incubation, but the nuclear RNA showed a rapid initial rate of incorporation that rapidly decreased to zero. In contrast, all of the cytoplasmic fractions showed almost no incorporation in the earliest time periods while the rate accelerated with time. The data were compatible with an incorporation of label in the nucleus with subsequent movement into the cytoplasm. In subsequent experiments fluoro-orotic acid (5 micromoles) was added to slices after 1 or 2 hours incubation with

labeled orotic acid (1 micromole) and the overall rate of incorporation into RNA of the slices dropped to zero immediately. However labeled RNA diminished in the nuclear fraction and increased in the cytoplasmic fractions. This experiment suggested transfer of RNA from nucleus to cytoplasm since it was proved that no increase in label occurred after the addition of fluoro-orotic acid. Another approach to the problem was attempted by Schneider, Scholtissek and Potter (cf. Schneider[58]). Rat liver nuclear RNA was labeled in vivo, and the isolated nuclei were incubated in non-radioactive cytoplasm. Labeled RNA moved from nuclei to cytoplasm without passing through an acid-soluble form.

Without attempting to review all of the relevant papers on this subject, it may be said that we prefer to assume for the moment that some RNA is synthesized in the nucleus and some is synthesized in the cytoplasm along lines suggested in Fig. 7.2, and that the relative proportions probably differ for different EFS systems, which in turn will vary according to type of cell and cell environment.

REFERENCES

1. ACS, HARTMAN, BOMAN, and LIPMANN. Fed. Proc. 18:700 (1959).
2. ADLER and GUTMAN. Science 130:862 (1959).
3. BEERS. Biochem. J. 66:686 (1957).
4. BELTZ, VAN LANCKER and POTTER. Cancer Research 17:688 (1957).
5. BOLLUM. J. Am. Chem. Soc. 80:1766 (1958).
6. BOLLUM. J. Biol. Chem. 234:2733 (1959).
7. BOLLUM. Fed. Proc. 19:305 (1960).
8. BOLLUM. J. Biol. Chem. 235:PC18 (1960)
9. BOLLUM, ANDEREGG, McELYA, and POTTER. Cancer Research 20:138 (1960).

10.   *BOLLUM and POTTER. J. Am. Chem. Soc. 79:7603
         (1957).*
11.   *BOLLUM and POTTER. J. Biol. Chem. 233:478
         (1958).*
12.   *BOLLUM and POTTER. Cancer Research 19:561 (1959).*
13.   *BOLTON, BRITTEN, COWIE, McCARTHY, McQUILL-
         EN and ROBERTS. Carnegie Institution of
         Washington Year Book 58, page 259 (1959).*
14.   *CANELLAKIS. Biochim. Biophys. Acta 23:217 (1957).*
15.   *COHN. J. Biol. Chem. 235:1488 (1960).*
16.   *DAVIDSON, SMELLIE, KEIR and McARDLE. Nature
         182:589 (1958) and SMELLIE, KEIR and DAVID-
         SON (I) Biochim. Biophys. Acta 35:389 (1959);
         KEIR and SMELLIE (II) ibid. 35:405 (1959);
         SMELLIE, GRAY, KEIR, RICHARDS, BELL,
         and DAVIDSON (III) ibid. 37:243 (1960).*
17.   *DAVIS, CARLUCCI and ROUBEIN. J. Biol. Chem.
         234:1525 (1959).*
18.   *DUNN. Biochim. Biophys. Acta 34:286 (1959).*
19.   *EDMUNDS and ABRAMS. Biochim. Biophys. Acta.
         26:227 (1957).*
20.   *GOLDWASSER. J. Am. Chem. Soc. 77:6083 (1955).*
21.   *GRIFFIN, TODD and RICH. Proc. Nat. Acad. Sci.
         44:1123 (1958).*
22.   *GRUNBERG-MANAGO and OCHOA. J. Am. Chem.
         Soc. 77:3165 (1955).*
23.   *GRUNBERG-MANAGO, ORTIZ, and OCHOA. Biochim.
         Biophys. Acta 20:269 (1956).*
24.   *HARRIS. Biochem. J. 73:362 (1959); 74:276 (1960).*
25.   *HECHT and POTTER. Cancer Research 16:988 (1956).*
26.   *HECHT and POTTER. Cancer Research 16:999 (1956).*
27.   *HECHT and POTTER. Cancer Research 18:186 (1958).*
28.   *HECHT, STEPHENSON and ZAMECNIK. Biochim.
         Biophys. Acta. 29:460 (1958).*
29.   *HECHT, STEPHENSON and ZAMECNIK. Proc. Nat.
         Acad. Sci. (U.S.) 45:505 (1959).*
30.   *HECHT, ZAMECNIK, STEPHENSON and SCOTT. J.
         Biol. Chem. 233:954 (1958).*

31. HEIDELBERGER, HARBERS, LEIBMAN, TAKAGI and
         POTTER. Biochim. Biophys. Acta. 20:445 (1956).
32. HERBERT. J. Biol. Chem. 231:975 (1958).
33. HERBERT. J. Biol. Chem. 225:659 (1957).
34. HIATT and BOJARSKI. Biochem. Biophys. Res. Comm.
         2:35 (1960).
35. HOAGLAND in Chargaff and Davidson 3:in press (1960).
36. HOAGLAND, STEPHENSON, SCOTT, HECHT and
         ZAMECNIK. J. Biol. Chem. 231:241 (1958).
37. HURLBERT and POTTER. J. Biol. Chem. 195:257
         (1952).
38. HURLBERT and POTTER. J. Biol. Chem. 209:1
         (1954).
38a. HURWITZ. J. Biol. Chem. 234:2351 (1959).
39. KORNBERG series:
     a. KORNBERG, LEHMAN, BESSMAN, and SIMMS.
         Biochim. Biophys. Acta. 21:197 (1956).
     b. KORNBERG in McElroy and Glass. The Chemical
         Basis of Heredity. Johns Hopkins Press p. 579
         (1957).
     c. (I) LEHMAN, BESSMAN, SIMMS and KORNBERG.
         J. Biol. Chem. 233:163)(1958).
     d. (II) BESSMAN, LEHMAN, SIMMS and KORNBERG.
         J. Biol. Chem. 233:171 (1958).
     e. (III) BESSMAN, LEHMAN, ADLER, ZIMMERMAN,
         SIMMS, and KORNBERG. Proc. Nat. Acad. Sci.
         44:633 (1958).
     f. (IV) ADLER, LEHMAN, BESSMAN, SIMMS and
         KORNBERG. Proc. Nat. Acad. Sci. 44:641
         (1958).
     g. (V) LEHMAN, ZIMMERMAN, ADLER, BESSMAN,
         SIMMS, and KORNBERG. Proc. Nat. Acad. Sci.
         44:1191 (1958).
     h. (VI) KORNBERG, ZIMMERMAN, KORNBERG and
         JOSSE. Proc. Nat. Acad. Sci. 45:772 (1959).
40. KHORANA in Chargaff and Davidson Vol. III, in press,
         1960.
41. LITTAUER and KORNBERG. J. Biol. Chem. 226:1077
         (1957).

42. *MANTSAVINOS and CANELLAKIS. J. Biol. Chem.*
         234:628 (1959).

43. *MII and OCHOA. Biochim. Biophys. Acta 26:445 (1957).*

44. *OCHOA. Arch. Biochem. Biophys. 69:119 (1957).*

45. *OCHOA and HEPPEL in McElroy and Glass. The*
         Chemical Basis of Heredity. The Johns Hopkins
         Press, Baltimore p. 615 (1957).

46. *OLMSTEAD and LOWE. J. Biol. Chem. 234:2965,*
         2971 (1959).

47. *OSAWA and OTAKA. Biochim. Biophys. Acta 36:549*
         (1959).

48. *PATERSON and LePAGE. Cancer Research 17:409*
         (1957).

49. *PLAUT and RUSTAD. Biochim. Biophys. Acta 33:59*
         (1959).

50. *POTTER, HECHT and HERBERT. Biochim. Biophys.*
         Acta 20:439 (1956).

51. *PREISS, BERG, OFENGAND, BERGMANN, and*
         DIECKMANN. Proc. Nat. Acad. Sci. U.S.
         45:319 (1959).

52. *PRESCOTT. J. Biophys. Biochem. Cytol. 6:203 (1959).*

53. *ROBERTS, editor, Microsomal Particles and Protein*
         Synthesis. Pergamon Press, 1958.

54. *ROBERTS. Rev. Modern Physics 31:170 (1959).*

55. *ROBERTS in Zirkle, A Symposium on Molecular Biol-*
         ogy. U. of Chicago Press, p. 201 (1959).

56. *SCHLENK in Chargaff and Davidson Vol. II page 309*
         (1955).

57. *SCHMITZ, HURLBERT, and POTTER. J. Biol. Chem.*
         209:41 (1954).

58. *SCHNEIDER. J. Biol. Chem. 234:2728 (1959).*

59. *SCHNEIDER and POTTER. J. Biol. Chem. 233:154*
         (1958).

60. *SINGER. J. Biol. Chem. 232:211 (1958).*

61. *SINGER, HEPPEL and HILMOE. Biochim. Biophys.*
         Acta 26:447 (1957). J. Biol. Chem. 235:738
         (1960).

62. *SINGER, HILMOE and HEPPEL. J. Biol. Chem.*
         235:751 (1960).

63. SMELLIE in Chargaff and Davidson. Vol. II, p. 393 (1955).

64. SMITH, CORDES, and SCHWEET. Biochim. Biophys. Acta 33:286 (1959).

65. TISSIERES, WATSON, SCHLESSINGER and HOLLING-WORTH. J. Mol. Biology 1:221 (1959).

66. WEISS and GLADSTONE. J. Am. Chem. Soc. 81:4118 (1959).

67. ZACHAU, ACS, and LIPMANN. Proc. Nat. Acad. Sci. 44:885 (1958).

68. ZALOKAR. Nature 183:1330 (1959).

Chapter VIII
Catabolic Pathways
Competitive to Biosynthesis

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I.   *ALTERNATIVE METABOLIC PATHWAYS*

Catabolism is the term applied to chemical degrada-
tions that occur in living organisms. It is considered
to be the opposite of anabolism, which is the term ap-
plied to the biosynthetic reactions. Our purpose here
is to show the chemical pathways for purine and pyrim-
idine catabolism and to show how these pathways are
connected to the anabolic pathways. The catabolism
of the carbohydrate moieties of the nucleic acids quick-
ly merges with carbohydrate metabolism in general
and will be only briefly mentioned.

The headwaters of the catabolic streams arise in the
foothills of the biosynthetic landscape - a series of
compounds that represent the first steps in the degra-
dation of the acid soluble nucleotides, i.e., the purine
and pyrimidine ribosides and deoxyribosides, which
arise by the dephosphorylation of the corresponding
phosphates. With the apparent exception of cytosine,
each of the nucleosides is in reversible equilibrium
with its corresponding base through the action of nu-
cleoside phosphorylases, as follows:

Purine or pyrimidine ribo- or deoxyribonucleoside $\rightleftharpoons$
  ribose- or deoxyribose-1-phosphate + purine or pyrimidine base   (1)
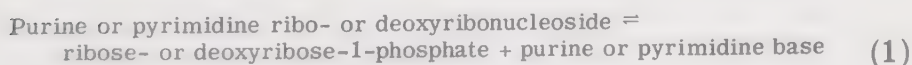
We regard this type reaction as neither catabolic nor
anabolic but as a sort of equalizer or shock-absorber

in the interplay between catabolic and anabolic reactions[8]. The compounds on each side of the equilibrium participate in a variety of reactions and the flow from one side to the other will depend on the fate of the reactants in reactions outside the system shown. The alternatives pursued by these reactants will now be discussed in a general way.

The nucleosides in general arise from the corresponding nucleotides by the action of phosphatases. Segal and Brenner[23] have recently studied the 5'-nucleotidases of rat liver microsome fraction and reported the following relative rates of dephosphorylation: AMP, 100; UMP, 67; CMP, 55; IMP, 27; GMP, 26; and dAMP, 10. The 2'(3') phosphates were not dephosphorylated, and they probably do not occur as normal acid soluble components. The action of the phosphatases is essentially irreversible, but the nucleosides can be converted back to the nucleotides by the action of phosphokinases, which utilize the terminal phosphate of ATP for the formation of the nucleotides. Each of the two reactions is essentially irreversible and the result is a cycle that is available for interconversions:

de novo synthesis → ribo- or deoxyribonucleotides ← preformed bases

(phosphatases) ↓   ↑ (phosphokinases + ATP)

orthophosphate + ribo- or deoxyribonucleosides ⇌ bases + sugar
                                                        phosphates   (2)
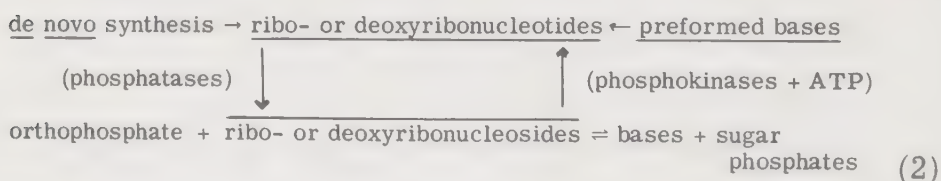
If this cycle turned over very rapidly it would be a very wasteful operation. All indications point to a very slow turnover, and the respective reactions are probably separated into different compartments in the cell[23]. The sluggishness of the turnover in rat liver in vivo was demonstrated by Brumm, Siekevitz and Potter[2] who injected P^{32} in the form of inorganic phosphate and isolated AMP, GMP, CMP and UMP and the corresponding di- and triphosphates. All of the latter were rapidly labeled and were in equilibrium with each other, but the monophosphates picked up P^{32} very

slowly, and the incorporation that was seen must have
been partly explained by de novo pathways. In the
cycle shown, the phosphatases must be considered as
catabolic in action, and an important site of competi-
tion between catabolism and anabolism thus appears:

Higher phosphates

(Anabolism: arrow pointing up)

Nucleoside monophosphates (3)

(Catabolism: arrow pointing down)
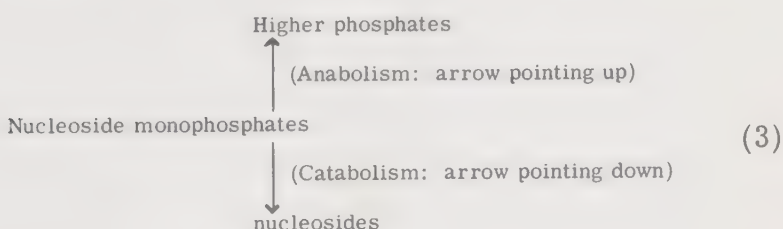
nucleosides

Similarly, we can describe competitive reactions for
the nucleosides as a segment of reaction (2):

nucleotides

(anabolism) (4)

nucleosides = bases + sugar phosphates
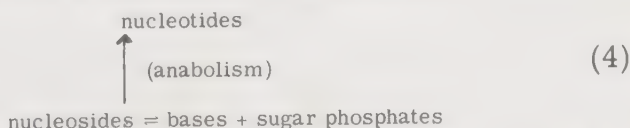
If we examine the fate of the bases in reaction (4) we
again find that alternative pathways demonstrating
competition between catabolism and anabolism can be
demonstrated:

Ribonucleoside monophosphates

(PRPP reaction)

(5)

bases

degradative products
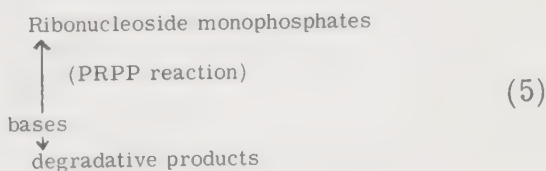
The detailed fate of individual bases will be described
below.

The fate of the sugar phosphates must be described
separately from each other. Ribose-1-phosphate has
clearly defined alternatives (Chapter V):

PRPP

(6)

(ATP)

ribonucleosides = R-1-P = R-5-P = glycolytic intermediates
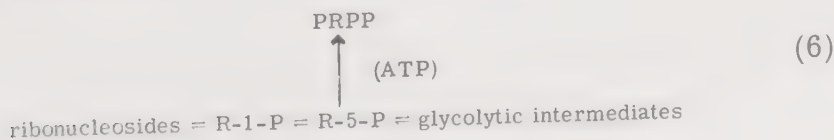
In the case of deoxyribose-1-phosphate the situation is less clear:

$$\text{deoxyribonucleosides} \rightleftharpoons \text{dR-1-P} \rightleftharpoons \text{dR-5-P} \atop \qquad\qquad\qquad\qquad\qquad\quad \downarrow \atop \qquad\qquad\qquad\qquad\qquad \text{degradation only (?)} \qquad (7)$$
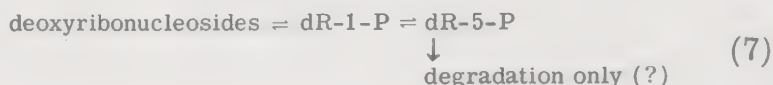
The exact intermediates in the degradation of dR-5-P are not established but may proceed by way of acetaldehyde and glyceraldehyde-3-phosphate in a reversal of the Racker reaction (Chapter V). Boxer and Shonk[1] have shown that this degradative pathway is diminished in regenerating liver and in Novikoff hepatoma compared to normal rat liver.

Reactions (2) to (7) show that at nearly every step of metabolism we find compounds confronted with alternative pathways that are catabolic versus pathways that are anabolic. These pathways will now be considered in greater detail. In every segment of the pathways shown, the ratios of the competing enzymes differ from one type of base to another and from one type of cell to another, and these differences can be indicated to a very limited extent at the present time.

II. THE CATABOLISM OF THE PURINES

Purine catabolism follows the type reactions shown in equations (2) to (5) above, and in addition there are interconversions between purines at the level of the monophosphates, the ribosides, and the free bases, as shown in Fig. 8.1.

The interconversions at the monophosphate level come to a focus at IMP, which can be converted to AMP and GMP and be formed from them by the action of different enzymes (Chapter VI). Each of the monophosphates can be dephosphorylated to the nucleoside.
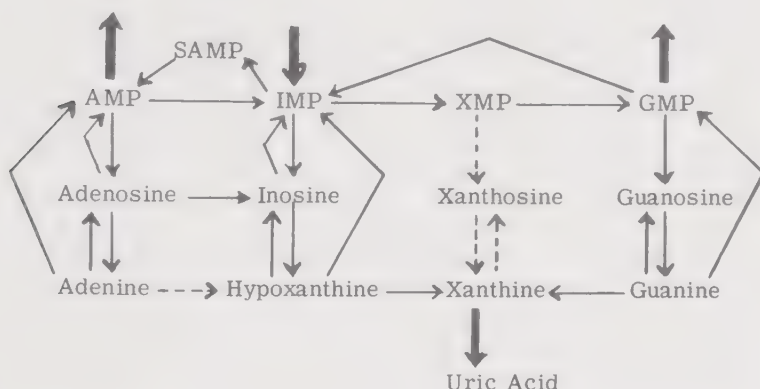
Fig. 8.1. Catabolism of the purines. Reactions leading to uric acid. IMP is
the entrance leading from the <u>de novo</u> pathway, while AMP and
GMP are the exits to the anabolic pathways and xanthine is the exit
to the catabolic pathway. Abbreviations and structures as in Chap-
ters II and V. The dashed line arrows are used to indicate pathways
that are weak or missing in comparison with other pathways. The
bent arrows are used to indicate that reversal is accomplished by a
different enzyme system, usually coupled with an energy donor.

The nucleosides follow the type reaction (4) and in
addition there is a great deal of evidence showing the
occurrence of an enzyme that deaminates adenosine to
inosine and probably also acts on the deoxyriboside[24].
Evidence for the deamination of guanosine is less clear.

At the level of the free bases, adenine appears to lack
a deaminase in the animal body, while guanine is de-
aminated to xanthine[24, 25]. Hypoxanthine is readily con-
verted to xanthine in rat liver and in pigeon kidney and
xanthine is converted to uric acid. As suggested by
Fig. 8.1, xanthine is the exit to the catabolic pathway,
and is a critical "point of no return" for purines moving
in the direction of catabolism in higher organisms.

In man and other primates, and in birds and terrestial
reptiles, uric acid is the end product of purine metab-
olism, but in mammals other than the primates, allan-
toin is the chief end product. The intermediates

between uric acid and allantoin have been the subject
of considerable study by Canellakis and others[5, 6, 7, 16].
Uric acid is first oxidized to a symmetrical product
with no loss of CO_2. This product is probably hydrox-
yacetylene-diureine-carboxylic acid (HDC) which is
hydrolyzed to 5-ureido-2-imidazole-4, 5-diol-4-
carboxylic acid (UIDC), also referred to as 'new'
uroxanic acid, which loses the No. 6 carbon of the pu-
rine ring as CO_2 to form allantoin hydrate and finally
allantoin. These reactions and the further conversions
to allantoic acid, urea, and ammonia that occur in
various marine organisms[25] are shown in Fig. 8.2.
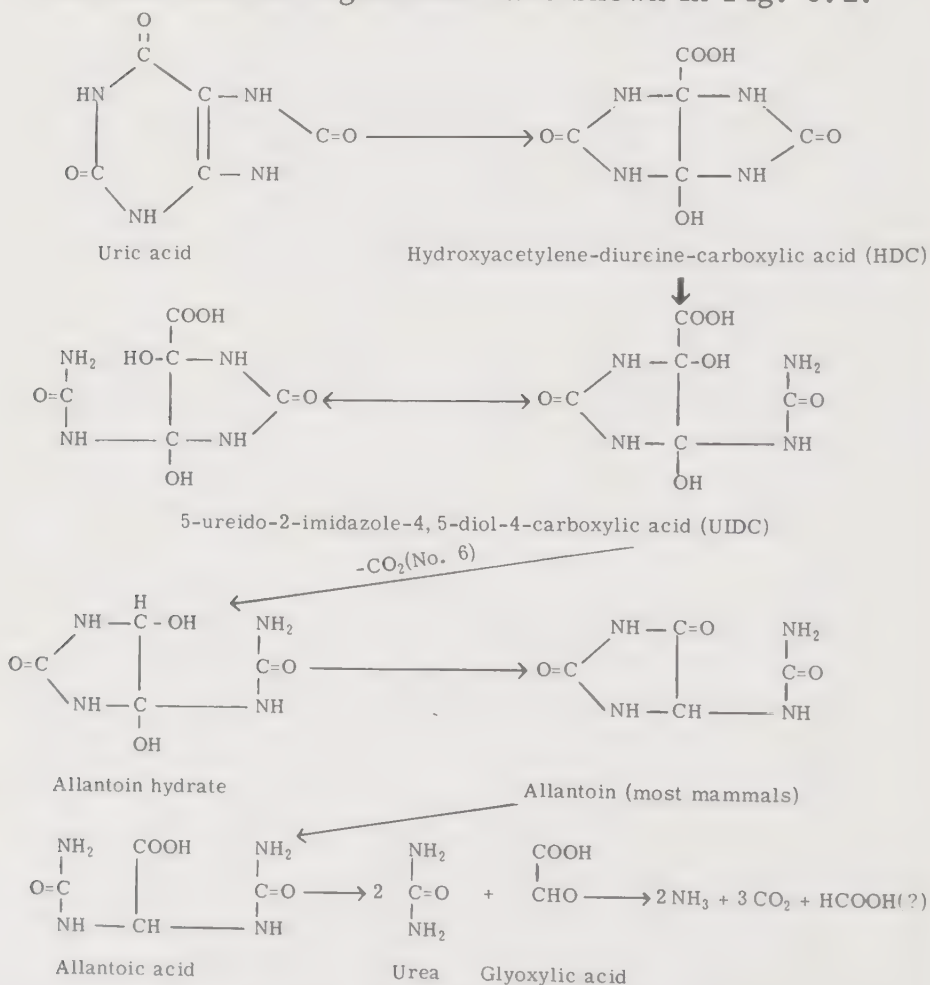
Fig. 8.2. Catabolism of the purines. Breakdown of uric acid.

The two forms of UIDC shown in Fig. 8.2 are in equilibrium with each other and can lead to the distribution of C^{14} from the 2-position in uric acid between the products of carbons 2 and 8 in allantoin. However Canellakis et al[5, 6] showed that in the presence of borate, which evidently combined preferentially with the form having cishydroxyl groups, the decomposition proceeded asymmetrically as in Fig. 8.3, so that uric acid labeled in position 2 yielded alloxanic acid with all of the C^{14} from the uric acid and none of the C^{14} in urea. Conversely, when they started with uric acid labeled in position 8, all of the C^{14} was recovered in urea and none was in alloxanic acid[5, 6].

Uric acid -2-C^{14} ⟶ HDC ⟶ UIDC

```
              COOH                                      COOH
               |                                         |
    NH ——— C ——— O                             NH ——— C - OH                NH₂
   /        |                                 /         |                    |
O=C*        |   B-OH  ———→   O=C*            |           +           C=O
   \        |                                 \         |                    |
    NH ——— C ——— O                             NH ——— C=O                NH₂
               |
          NH-CO-NH₂
```

Borate complex of UIDC   Alloxanic acid   Urea
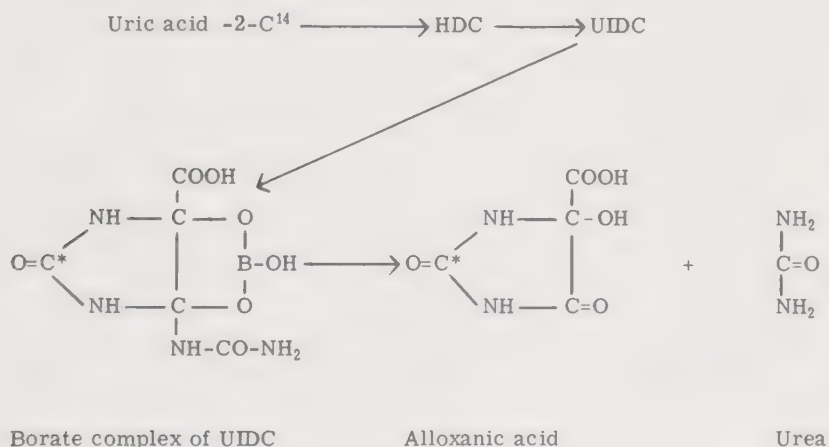
Fig. 8.3. Catabolism of the purines. Breakdown of uric acid by uricase in the presence of borate buffer without liberation of carbon No. 6 as CO_2. Compare with Fig. 8.2.

At present there is no evidence to suggest that the above products of purine catabolism can be salvaged at the level of xanthine or beyond, although considerable utilization of preformed nucleosides and bases is possible at the stages preceding xanthine as shown in Fig. 8.1.

Little has been said about bacterial metabolism of the purines in the above discussion. Recent studies by Mans and Koch[17] showed that E. coli rapidly converted adenosine and deoxyadenosine into inosine and hypoxanthine deoxyriboside in the medium and converted each of these to free hypoxanthine in the medium, which was then reutilized for nucleic acid synthesis. Adenine was also utilized for nucleic acid synthesis, apparently without conversion to hypoxanthine. It thus was indicated that hypoxanthine and adenine were not catabolized in this organism, and this is probably a general rule for most micro-organisms unless selected under conditions that pick mutants or strains able to utilize these compounds as an energy source. Rabinowitz and colleagues have carried out extensive studies with such organisms and have shown detailed schemes[21] for the fermentation of xanthine leading to the degradative formation of several of the intermediates in the de novo synthesis of the purines rather than any of the compounds indicated in Fig. 8.2. However the products of xanthine catabolism appeared to lack the ribose phosphate moiety that is a part of all of the de novo intermediates (Chapter VI). Whether enzymes are available for the ribotidation of these compounds in various organisms is not yet clear.

III.   THE CATABOLISM OF THE PYRIMIDINES

Pyrimidine catabolism follows the type reactions shown in equations (2) to (5) in section I, except for the special case of cytosine, which is apparently not vigorously metabolized in any direction. Fig. 8.4 shows these reactions in greater detail for each of the pyrimidines and in addition shows the rather specific pathways which interconversions follow. It appears at present that the catabolism of cytosine derivatives occurs by way of uracil derivatives, and that the problem in pyrimidine catabolism is the study of the fate of uracil and thymine. This figure is mainly relevant to the

mammal as a whole. A recent report shows a some-what similar pattern in E. coli[15] except that the degradation of uracil and thymine were not prominent.

UMP → CMP → dCMP → dUMP → TMP

uridine ← cytidine   deoxycytidine   deoxyuridine   thymidine

cytosine   thymine
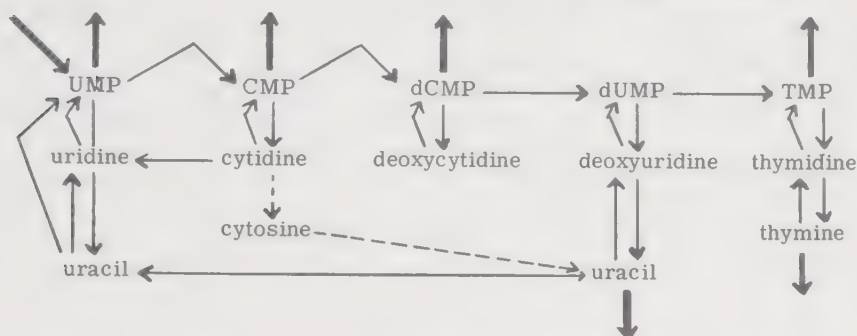
uracil ←   uracil

Fig. 8.4.   Catabolism of the pyrimidines.   Reactions leading to uracil and thymine.   UMP is the entrance leading from the de novo pathway; UMP, CMP, dCMP, and TMP are exits leading to anabolic pathways; and uracil and thymine are exits leading to the catabolic pathways.   Bent arrows as in Fig. 8.1 to indicate reactions that require an energy donor not indicated in the scheme.

The catabolism of uracil and thymine was studied in selected bacteria by Hayaishi and Kornberg[11], by Wang and Lampen[28] and by Lara[14].   Cytosine and 5-methyl-cytosine were deaminated to uracil and thymine and these two compounds were oxidized to barbituric acid and 5-methylbarbituric acid respectively, i.e. the 2:4-dihydroxypyrimidines were oxidized to the corresponding 2:4:6-trihydroxypyrimidines.   It was also shown that barbituric acid was broken down to urea and malonic acid.   These pathways are in contrast to the reductive pathway found in animal tissues, where the reaction occurs almost exclusively in the liver and at a much slower rate in the kidney.

The catabolism of uracil and thymine in animals has been studied by Fink, Cline, Henderson and Fink[9] using liver slices and by Canellakis[3] using liver homogenates and cell fractions.   On the basis of these and earlier studies it is clear that the main catabolic pathway in animals is reductive in the initial stages as shown in Fig. 8.5.   It seems probable that the distinc-

tion between the oxidative and the reductive pathways
is not absolute, but that vestiges of each may be found
in the presence of the other, with further research.

$$
\begin{array}{cc}
& O \\
& \| \\
& C \\
HN \diagup \quad \diagdown CH \\
| \qquad \| \\
O=C \diagdown \quad \diagup CH \\
N \\
H
\end{array}
\qquad
\begin{array}{cc}
& O \\
& \| \\
& C \\
HN \diagup \quad \diagdown C\text{-}CH_3 \\
| \qquad \| \\
O=C \diagdown \quad \diagup CH \\
N \\
H
\end{array}
$$

Uracil (U)   Thymine (T)

⇅ 2H   ⇅ 2H

$$
\begin{array}{cc}
& O \\
& \| \\
& C \\
HN \diagup \quad \diagdown CH_2 \\
| \qquad | \\
O=C \diagdown \quad \diagup CH_2 \\
N \\
H
\end{array}
\qquad
\begin{array}{cc}
& O \\
& \| \\
& C \\
HN \diagup \quad \diagdown CH\text{-}CH_3 \\
| \qquad | \\
O=C \diagdown \quad \diagup CH_2 \\
N \\
H
\end{array}
$$

Dihydrouracil (DHU)   Dihydrothymine (DHT)

⇅   ⇅

$$
\begin{array}{cc}
& COOH \\
& | \\
NH_2 \quad CH_2 \\
| \qquad | \\
O=C \diagdown \quad \diagup CH_2 \\
N \\
H
\end{array}
\qquad
\begin{array}{cc}
& COOH \\
& | \\
NH_2 \quad CH\text{-}CH_3 \\
| \qquad | \\
O=C \diagdown \quad \diagup CH_2 \\
N \\
H
\end{array}
$$

β-ureidopropionic acid (BUP) β-ureidoisobutyric acid (BUIB)

⇅   ⇅

$$
\begin{array}{c}
COOH \\
| \\
CH_2 \\
| \\
CH_2 \\
| \\
NH_2
\end{array}
\qquad
\begin{array}{c}
COOH \\
| \\
CH\text{-}CH_3 \\
| \\
CH_2 \\
| \\
NH_2
\end{array}
$$

β-alanine (BAL) β-aminoisobutric acid (BAIB)

⇅   ⇅

$$
\begin{array}{cc}
COOH & COOH \\
| & | \\
CH_2 \rightleftharpoons CH_2 \\
| & | \quad (BHP) \\
CHO & CH_2OH
\end{array}
\qquad
\begin{array}{cc}
COOH & COOH \\
| & | \\
CH\text{-}CH_3 \rightleftharpoons CH\text{-}CH_3 \\
| & | \quad (HIB) \\
CHO & CH_2OH
\end{array}
$$

Malonic semialdehyde (MSA)   Methylmalonic semialdehyde (MEMSA)
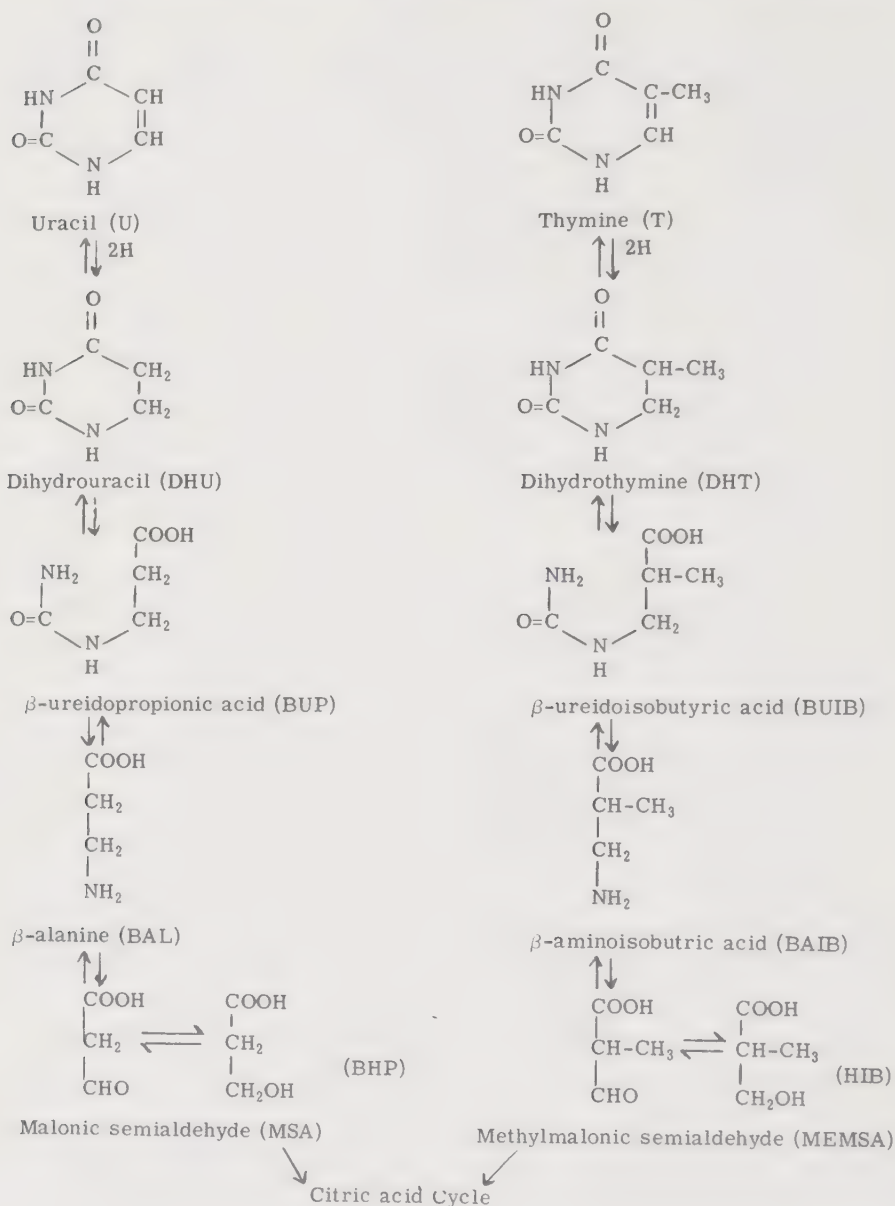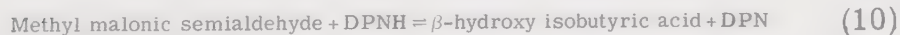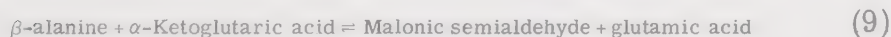
↘   Citric acid Cycle   ↙

Fig. 8.5. Catabolism of the pyrimidines. Reductive cleavage of uracil and thymine.
Abbreviations (BHP) and (HIB) stand for β-hydroxypropionic acid and β-
hydroxyisobutyric acid, respectively. Coenzymes and cosubstrates omitted,
see text.

The reductive steps were shown by Canellakis[3] to re-
quire TPNH in the case of both thymine and uracil. It
is not yet known whether a single enzyme acts on both
thymine and uracil or whether there is a specific en-
zyme for each, and until purified enzyme preparations
are available it would be premature to conclude that
the coenzyme requirements are established. Wallach
and Grisolia[27] studied the enzymatic conversion of the
hydropyrimidines to the β-ureido compounds using a
purified enzyme from calf liver and found the reaction
strongly dependent upon pH, with an optimum for the
reverse reaction (ring closure) at pH 5.5 and an opti-
mum for opening the dihydrothymine ring at pH 8.5
and the dihydrouracil ring at pH 10.0. Fritzson[10]
studied the relative rates of the degradative steps for
uracil in rat liver slices and reported the rates in
μmoles per gram per hour as follows:

$$ U \xrightarrow{0.30} DHU \xrightarrow{26} BUP \xrightarrow{20} BAL \xrightarrow{0.06} CO_2 $$

(See Fig. 8.5)

Studies on the fate of the β-amino end products are
found in the work of Kupiecki and Coon[13] who showed
that these compounds are also products of valine and
propionic metabolism. The following reversible re-
actions were described:

β-aminoisobutyric acid and α-Ketoglutaric $\rightleftharpoons$ Methylmalonic semialdehyde   (8)
                                                        + glutamic acid

β-alanine + α-Ketoglutaric acid $\rightleftharpoons$ Malonic semialdehyde + glutamic acid   (9)

Methyl malonic semialdehyde + DPNH $\rightleftharpoons$ β-hydroxy isobutyric acid + DPN   (10)

Reaction (10) appears to be somewhat analogous to the
reduction of pyruvate to lactate, and the oxidative fate
of the two semi-aldehydes appears to involve conver-
sion to acyl-CoA derivatives in a further analogy with
the oxidation of pyruvate. Yamada and Jakoby[29] have
reported the direct conversion of malonic semialdehyde

to acetyl-CoA, and an analogous conversion of methyl-malonic semialdehyde to methylmalonyl-CoA or to propionyl-CoA might be anticipated. New developments in the field of propionate metabolism include methyl-malonic acid, succinic acid, and propionic acid all as acyl derivatives of Coenzyme A, the role of biotin, and DMBC (a coenzyme form of vitamin B_{12}), as reported by Stadtman et al[26]. The following reactions were among those reported:

$$\text{propionyl-CoA} + CO_2 \text{ biotin enzyme} \rightleftharpoons \text{biotin enzyme} + \text{methyl malonyl-CoA} \quad (11)$$

$$\text{methylmalonyl-CoA} \xrightleftharpoons[\quad]{\text{DMBC}} \text{succinyl-CoA} \quad (12)$$
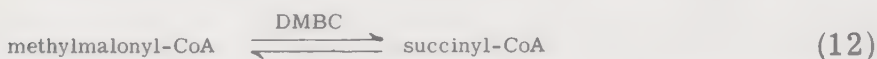
From these examples it would appear that methylmalo-nyl-CoA is a highly reactive intermediate. It seems possible that methylmalonic semialdehyde is directly converted to methylmalonyl-CoA in the presence of a hydrogen acceptor, and that this compound can be converted either to propionyl-CoA or succinyl-CoA, incidentally serving as the intermediate between these two compounds in systems carrying on the interconversion. Unpublished data in the author's laboratory has shown that label from methyl-labeled thymine can be picked up in the 4-carbon dicarboxylic acids of the citric acid cycle in liver at least as early as 30 minutes after injection in rats. The pivotal position of methylmalonyl CoA is illustrated in Fig. 8.6.

The concept that catabolic pathways in higher organisms might be important mechanisms for the control of growth was proposed many years ago by Potter and reiterated on various occasions (cf.[20]) in relation to the cancer problem (Chapter XVI). It has received much support from studies on the pyrimidines, especially with labeled uracil. It was originally reported by Plentl and Schoenheimer that uracil was ineffective as a nucleic acid precursor, but studies by Rutman et al[22] and by Canellakis[4] showed that uracil could be used for the synthesis of RNA if the system for degradation was loaded beyond its capacity.

```
COOH                        COOH
 |        Malonic            |        Methylmalonic
CH₂       Semialdehyde      CH-CH₃    Semialdehyde
 |                           |
C=O                         C=O
H                           H
    +                           +
H                           H                              COOH
S-CoA                       S-CoA                           |
                                                           CH₂
    ↓                           ↓                           |
                                                           CH₂      Succinyl
COOH                        COOH         B₁₂-coenzyme        |       -CoA
 |                           |        ⇌                   C=O
CH₂          Methyl         CH-CH₃                          |
 |           Malonyl         |                            S-CoA
Malonyl     C=O  -CoA        C=O
-CoA         |               |
            S-CoA           S-CoA
            ↑↓              ↑↓  +biotin enzyme
            CH₃             CH₂-CH₃
             |               |
            C=O             C=O
             |               |
            S-CoA           S-CoA

    Acetyl-Coa          Propionyl-CoA + CO₂-biotin enzyme
```
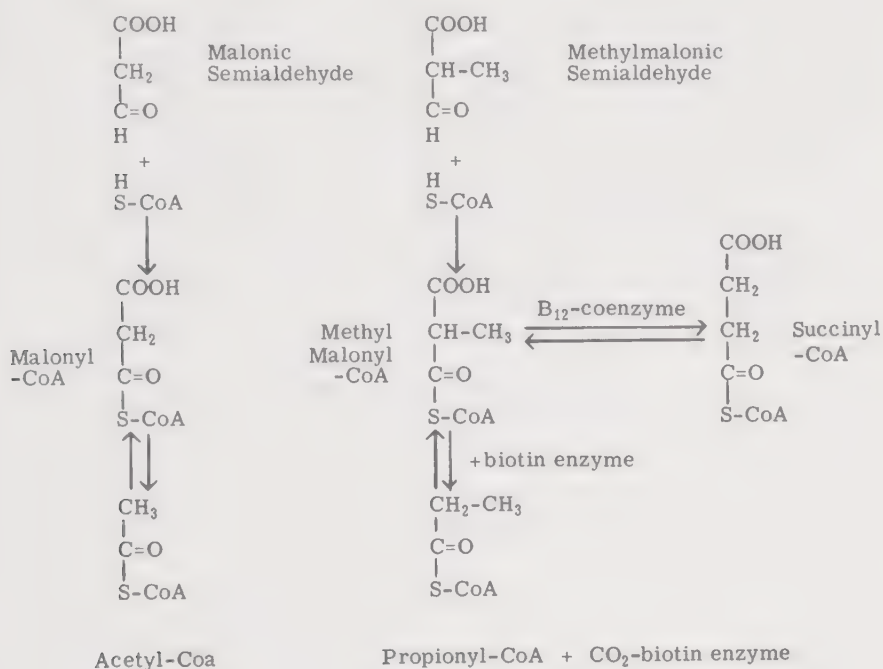
Fig. 8.6. Catabolism of the pyrimidines. Conversion of malonic semialdehyde to acetyl-CoA and conversion of methylmalonic semialdehyde to succinyl-CoA and to propionyl-CoA. Acetyl-CoA and succinyl-CoA lead directly into the citric acid cycle. Compare with Fig. 8.5.

The literature does not appear to include any systematic studies of uracil or thymine degradation in animal tissues but unpublished studies by Ono and Potter using soluble supernatant fractions from homogenates, fortified with TPN, glucose-6-phosphate, and ATP, have shown that pyrimidine catabolism is almost exclusively limited to the liver, with a very small activity in kidney. Nevertheless, the liver is able to dispose of injected uracil or thymine very rapidly, with the No. 2 carbon largely converted to CO_2 within 2 hours even with doses of considerable magnitude (Morse and Potter unpublished). Similar results were obtained with thymidine.

Further support for the concept that catabolic activity helps to regulate anabolic activity of pyrimidine enzymes comes from the work of Hiatt and Bojarski[12] who showed that thymidylic kinase activity could be greatly increased in both liver and kidney by giving injections of thymidine every hour for five hours. It is noteworthy that the amount of thymidine injected greatly exceeded the catabolic capacity of the liver as established by Morse and Potter. It is inferred that in normal non-growing liver and kidney the level of thymidine is so low that it either fails to induce or fails to relieve the suppression of the enzyme-forming systems for thymidine kinase and sequential enzymes. Since the increases noted[12] are not established as new synthesis (versus activation or protection from destruction) it is not possible to foresee the final implications at this time.

The question of whether the intermediates in Fig. 8.5 represent catabolic "points of no return" has been considered by Mokrasch and Grisolia[18] who use the name carbamoyl-β-alanine in preference to that employed elsewhere[9] for β-ureido-propionic (Fig. 8.5). They found that the free compound was poorly utilized but that in the form of the ribotide it was readily converted to UMP. Further investigation of the question of naturally occurring ribosides, ribotides, deoxyribosides or deoxyribotides of the compounds shown in Fig. 8.5 and their possible transport and reutilization is needed. Studies by Mukherjee and Heidelberger[19] have revealed significant amounts of the intermediate breakdown products of fluorouracil in urine and in tissues other than liver. The products are analogous to those shown in Fig. 8.5, with a fluorine atom on the No. 5 carbon of uracil. Since each of the intermediate steps may proceed at a different rate than the corresponding normal reaction the studies with fluorouracil may provide clues as to interrelations between tissues that would ordinarily escape attention.

REFERENCES

1. BOXER and SHONK. J. Biol. Chem. 233:535 (1958).
2. BRUMM, POTTER and SIEKEVITZ. J. Biol. Chem.
        220:713 (1956)
3. CANELLAKIS. J. Biol. Chem. 221:315 (1956).
4. CANELLAKIS. J. Biol. Chem. 227:701 (1957).
5. CANELLAKIS and COHEN. J. Biol. Chem. 213:379,
        385 (1955).
6. CANELLAKIS, TUTTLE and COHEN. J. Biol. Chem.
        213:397 (1955).
7. CAVALIERI, TINKER and BROWN. J. Am. Chem.
        Soc. 71:3973 (1949).
8. De VERDIER and POTTER. J. Nat. Cancer Inst. 24:13
        (1960).
9. FINK, CLINE, HENDERSON, and FINK. J. Biol.
        Chem. 221:425 (1956).
10. FRITZSON. J. Biol. Chem. 226:223 (1957); 235:719
        (1960).
11. HAYAISHI and KORNBERG. J. Biol. Chem. 197:717
        (1952).
12. HIATT and BOJARSKI. Biochem. Biophys. Res.
        Comm. 2:35 (1960).
13. KUPIECKI and COON. J. Biol. Chem. 229:743 (1957).
14. LARA. J. Bact. 64:271, 279 (1952).
15. LICHTENSTEIN, BARNER, and COHEN. J. Biol.
        Chem. 235:457 (1960).
16. MAHLER, BAUM, and HÜBSCHER. Science 124:705
        (1956).
17. MANS and KOCH. J. Biol. Chem. 235:450 (1960).
18. MOKRASCH and GRISOLIA. Biochim. Biophys. Acta.
        34:165 (1959).
19. MUKHERJEE and HEIDELBERGER. J. Biol. Chem.
        235:433 (1960).
20. POTTER. Fed. Proc. 17:691 (1958).
21. RABINOWITZ et al. J. Biol. Chem. 218:175, 189
        (1956); 222:537 (1956).
22. RUTMAN, CANTAROW and PASCHKISS. Cancer
        Research 14:119 (1954). J. Biol. Chem. 210:321
        (1954).

23. *SEGAL and BRENNER. J. Biol. Chem. 235:471 (1960).*
24. *SCHMIDT in Chargaff and Davidson I: 555 (1955).*
25. *SMELLIE in Chargaff and Davidson II: 393 (1955).*
26. *STADTMAN, OVERATH, EGGERER, and LYNEN. Biochem. Biophys. Res. Comm. 2:1 (1960).*
27. *WALLACH and GRISOLIA. J. Biol. Chem. 226:277 (1957).*
28. *WANG and LAMPEN. J. Biol. Chem. 194:775, 785 (1952).*
29. *YAMADA and JAKOBY. J. Biol. Chem. 235:589 (1960).*

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Chapter IX
Synthesis and Function
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I. INTRODUCTION

In 1949 Waldo Cohn published the first of a series of papers on the use of ion-exchange resins for the separation of hydrolysis products of nucleic acids[24]. This method was modified in our laboratory in order to examine the acid-soluble nucleotides of animal tissues. The modification consisted of a procedure referred to as extended gradient elution, by which the eluting solution was automatically varied from a solution with the least eluting power (water) to a solution that would remove all adsorbed compounds from the ion exchange column[41]. The procedure of gradient elution was admirably suited to the examination of unknown mixtures. Since the proper eluent for the unknown constituents could not be decided beforehand, a procedure that employed gradient elution could rapidly develop the conditions for eluting each adsorbed compound. This has been accomplished by measuring the total phosphorus, total radioactivity, or total E_{260} units ($\underline{E}$ x $\underline{ml}$) in a sample before applying it to a column and then continuing the elution by various means until the total has been recovered. Further work is usually required to obtain individual compounds in pure form, but the preliminary fractionation is very useful.

The method has been widely applied to a variety of materials and various modifications have been developed[80]. No attempt will be made to review either the methodology or the individual applications, but two

recent methodological studies will be mentioned.
Bucher et al[96] have developed a remarkable micro-
adaptation of the original method. In this modification
of biopsy sample of human liver weighing 41 mg. and
containing 1.4 μ moles of total phosphorus was sepa-
rated into four hundred 140 μ l fractions and 32 "peaks"
were tentatively identified in terms of ultraviolet light
absorption, phosphorus content as low as 5×10^{-9}
micromoles, and position on the chromatogram.
Martonosi[70] has proposed an anion exchange column
using bicarbonate as the anion on the resin and bicar-
bonate for elution. It was demonstrated that labile
phosphate esters could be eluted without hydrolysis,
but no studies with complex natural mixtures were
reported.

In 1958 Henderson and LePage[36] reviewed some 310
papers dealing with "Naturally Occurring Acid-Soluble
Nucleotides" and reported over 110 separate nucleo-
tides isolated mainly by means of gradient elution
chromatography. They were all derived from the
basic nucleotide structures described in Chapter II
and contained a purine or a pyrimidine base, at least
one ribose or deoxyribose molecule, and at least one
phosphate. It is rather remarkable that from the time
of the discovery of the adenine nucleotides in 1927
until 1954, so much work was carried out on the
adenine nucleotides without discovering the nucleo-
tides of guanine, uracil, cytosine and thymine. The
explanation is not a simple one and involves not only
the relative amounts of the various nucleotides, and
the fact that biochemists concentrated on the best
source of ATP, which happens to be a poor source of
other nucleotides (see chromatogram of muscle[95]) but
it also depended on the development of ion exchange
chromatography, the widespread installation of
Beckman DU spectrophotometers, and C^{14} counting
facilities. Moreover, the lag periods coincide with
periods of war and coincident decline in fundamental
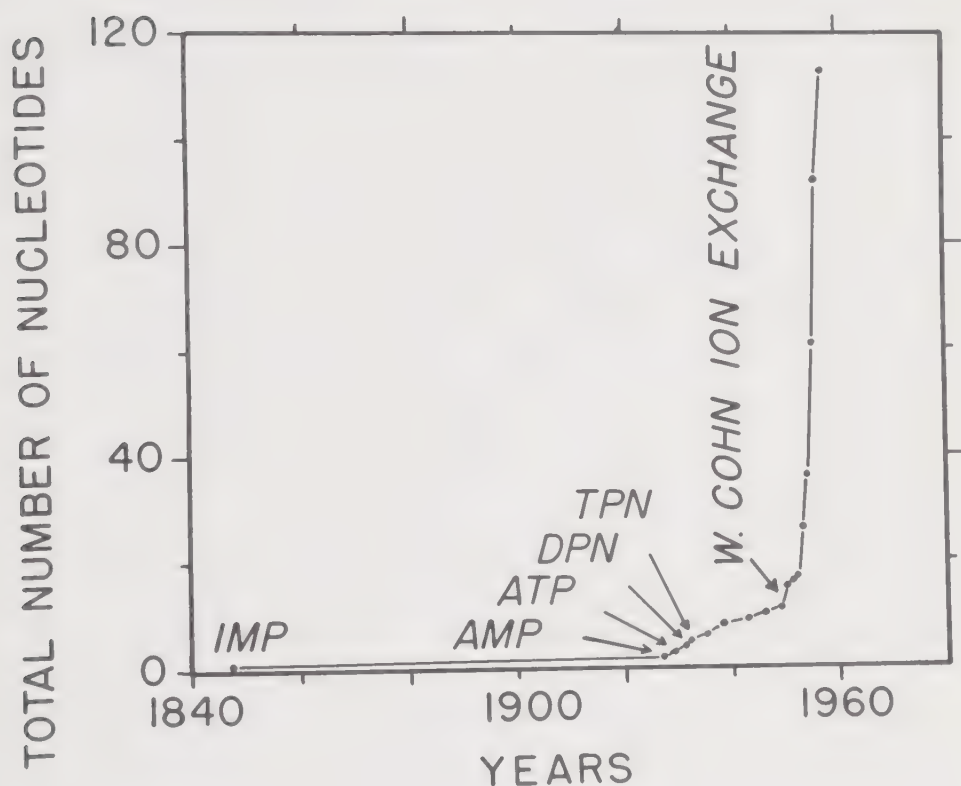research (see below). The long period during which

Fig 9.1. The discovery of acid-soluble nucleotides as a function of time. The cumulative total of individual nucleotides is shown. Based on data by Henderson and LePage[36]. Some compounds were known for many years before they were characterized, thus IMP, for example, was isolated in 1847 but its structure and phosphorus content were unknown until about 1928.[93] The structure of TPN was known before the structure of DPN, but DPN was known to contain adenylic acid before TPN had been discovered.[92, 92a] More recently the lag between discovery and characterization has been shorter so that the steep part of the curve is not greatly affected.

only a few nucleotides were known and the rapid up-
turn in their rate of discovery are shown in Fig. 9.1
which is based on the review by Henderson and
LePage[36]. Another useful review appeared at about
the same time by Baddiley and Buchanan[5], and con-
tains 164 references. It will be apparent from Fig.
9.1 that most of the new nucleotides were discovered
since 1954 and that for the last several years the list
has been growing at the rate of 20 to 25 nucleotides
per year.

The long lag period indicated in Fig. 9.1 goes back to
the discovery of IMP in 1847 by Liebig, but it was not
recognized at that time that the compound contained
phosphorus[36]. The story of the acid soluble nucleo-
tides can be traced back to Embden's laboratory in
1914[31] where an organic phosphate was associated with
adenine and ribose, but it was not until 1927 that
Embden and Zimmerman[32] obtained muscle AMP and
showed that it was not identical with the AMP that
Levine had obtained by hydrolysing yeast RNA. This
paper is very important in establishing the dichotomy
between the acid soluble nucleotides and the hydrolytic
products of the nucleic acids, a schism that was not to
be corrected for over 20 years. Muscle AMP (adeno-
sine 5'-phosphate) was shown to yield phosphate during
acid hydrolysis at a rate only 1/7 as fast as yeast AMP
(adenosine 3'-phosphate), and in 1928 Gerhard Schmidt[93]
showed that a deaminase from striated muscle was
very active on 5'-AMP but virtually without effect on
yeast AMP. The product was shown to be IMP and it
became clear that AMP was the source of IMP dis-
covered by Liebig in meat extract.

The characterization of muscle AMP and its distinc-
tion from yeast AMP was the key to the identification
of ATP in 1927 and the subsequent discoveries of the
adenosine-containing coenzymes DPN and TPN as
shown in Fig. 9.1[36]. In examining this chart and the
long lags between discoveries one cannot fail to be

impressed with the lag in Embden's laboratory 1914-1927 which coincides with World War I and the post-war years, and the lag from the adenine coenzymes, 1934-1935, to the uridine and other coenzymes in 1950 and following years, which marks the occurrence of World War II. The question of whether the ion exchange resins required a Manhattan project for their discovery and development can only be speculated upon, but if progress in nucleotide chemistry was in any way hastened by World War II technology, it was only the repayment of a debt long overdue.

The "nucleotide explosion" that occurred following the development of ion exchange columns led to the discovery of over a hundred different nucleotides in the space of a few years. It is not necessary to consider or to attempt to remember such a great number of nucleotides individually but it is worthwhile to note that they very definitely seem to follow certain rules and to fall into a rather limited number of categories. In general it appears that every nucleotide that occurs in a nucleic acid will also be found as the 5' monophosphate, diphosphate, and triphosphate in the acid soluble fraction and that in addition there are likely to be a series of compounds that can be looked upon as derivatives of these 3 forms and to possess coenzyme function. If we examine the converse form of this rule, we can suggest that if a nucleotide occurs as a 5' di- or triphosphate it is likely to occur in the form of nucleic acids and to have derivatives with coenzyme function. Inosinic acid, IMP, may be an exception to this rule since IDP and ITP are known, but do not appear to occur naturally in the form of derivatives or in nucleic acids; on the other hand, the natural occurrence of IDP and ITP may be restricted (see below). It should be pointed out that the occurrence of ribotides with 5'-phosphates is no guarantee that either polymers or coenzymes will be formed, and that apparently the di- and triphosphates must occur for this to be true. Thus the de novo pathways of purine and pyrimidine synthe-

sis include ribose 5'-phosphates but the higher phosphates of compounds such as glycine amide ribotide or orotidine-5'-phosphate have not been reported. Finally it should be emphasized that not all coenzymes are nucleotides.

The occurrence of a limited number of categories of nucleotides makes it possible to discuss them according to the nucleoside moiety (cf. Chapter II). All of the nucleotides found in "classical" RNA, i.e. AMP, GMP, CMP and UMP, have been found as the di- and triphosphates in the acid-soluble fraction of a number of animal tissues[94, 95] and all have been found in the form of coenzymes. Thus the discussion can logically be organized according to the nucleoside components. For more detailed duscussions, the student may consult The Enzymes, Volume 2[15] in which reviews on the acid-soluble nucleotides are similarly organized according to the 4 major nucleosides found in RNA. The adenosine nucleotides are reviewed by Bock[13], the uridine nucleotides by Leloir and Cardini[56], the cytidine nucleotides by Kennedy[47] and the guanosine and inosine nucleotides by Utter[107]. The discovery of thymidine nucleotides of various types is too recent and the documentation is too restricted to have been the subject of a review up to this time.

II.   NATURALLY OCCURRING ADENINE RIBONUCLEOTIDES

A.   AMP, ADP and ATP

The names and structures of twelve different unsubstituted adenine ribonucleotides are given in Table 9.1, using the system of structural shorthand introduced in Chapter VII. The first three compounds are AMP, ADP and ATP, compounds that have long been familiar to biochemists. The position of the ester phosphate on the 5'OH of the ribose moiety and the pyro-

phosphate nature of the remaining two phosphates has been established for many years[13, 36]. The

Table 9.1 NATURALLY-OCCURRING ADENINE MONONUCLEOTIDES *

	Structure **	Name and Abbreviation, if any	Reference
1.	p-r-A	Adenosine 5'phosphate (AMP)	1927 Embden and Zimmerman[32]
2.	ppp-r-A	Adenosine 5'-triphosphate (ATP)	1929 Lohmann[66]
3.	pp-r-A	Adenosine 5'-diphosphate (ADP)	1935 Lohmann[67]
4.	p-r-A / p-r-A	Diadenylic acid or Adenylic dinucleotide or Diadenosine diphosphate	1938 Kiessling and Meyerhof[49]
5.	pp-r-A / p-r-A	Diadenosine triphosphate	1938 Kiessling and Meyerhof[49] 1951 Ohlmeyer[74]
6.	ppp-r-A / pp-r-A	Diadenosine pentaphosphate	1932 Embden[30] 1934 Ostern[77]
7.	pppp-r-A	Adenosine tetraphosphate	1954 Marrian[69] 1955 Lieberman[59]
8.	ppppp-r-A	Adenosine pentaphosphate	1955 Sacks[90]
9.	r-A / p	Adenosine 3'-phosphate	1957 Doery[29]
10.	r-A / ppp	Adenosine 3'-triphosphate	1943 LePage and Umbreit[58]
11.	p-r-A / p	3', 5'-diphosphoadenosine (PAP)	1957 Gregory and Lipmann[33]
12.	p-r-A \/	3', 5'-Cyclic adenosine monophosphate	1958 Sutherland and Rall[105]

*The natural occurrence of deoxy-ATP has been reported by LePage[57].

**Structural shorthand based on system introduced in Chapter VII, Fig. 7.1.

A = adenine, r = ribose, p = phosphate, pp = pyrophosphate, p-r = ribose 5'-phosphate, r/p = ribose 3'phosphate.

review by Bock[13] describes recent advances in the physical chemistry of the adenine nucleotides and describes 11 classes of enzymatic reactions in which these nucleotides participate. For many years the adenosine nucleotides were the only known mechanisms by which energy could be gathered up from the processes of oxidation and glycolysis and funneled off into functional pathways of energy consumption. With the discovery of the other three types of nucleotides in the acid soluble fraction of animal tissues[94, 95] we carried out studies that attempted to answer the question of whether UTP, CTP and GTP could be generated by oxidative phosphorylation in mitochondria and came to the conclusion[37, 38] that ADP is probably the initial phosphate acceptor in oxidative phosphorylation with subsequent phosphorylation of UDP, CDP and GDP to the respective triphosphates by ATP. Subsequent studies using smaller particulates led to the same conclusion perhaps less ambiguously[27, 108]   In the case of the glycolytic phosphorylation associated with pyruvic kinase it was reported that all of the diphosphates, ADP, GDP, IDP, UDP and CDP could accept phosphate to form the triphosphates, but the relative activities were 100: 19: 12: 5: 2[101]. However the relative rates may indicate merely that the enzymes were fractionated as Utter has pointed out[108]. The values for GDP, IDP, UDP and CDP could be false and high due to contaminating transferases[28] or false and low due to partial elimination of specific pyruvic kinases. A vast amount of work remains to be done before it can be concluded that ADP is the specific acceptor for all of the reactions in which it has been demonstrated to take part, and it should be realized that the problem of what occurs in cells cannot be answered by testing the various diphosphates with purified enzymes that were fractionated on the basis of their ability to utilize ADP. The other horn of the dilemma is that conclusions with crude systems

can be misleading because a reaction between a phosphate donor and ADP may be direct, while transphosphorylating enzymes may be present to give the appearance of reaction with the other nucleotide diphosphates[37, 38]. The only ultimate solution is probably fractionation in which the assay is based on diphosphates other than ADP, or a measure of the ratios of activities with the various diphosphates throughout the course of the fractionation. There is no doubt that many of the reactions studied with ADP simply have not been tested with other diphosphates.

Despite the above uncertainties, the trend of opinion is that the adenosine nucleotides are the primary energy gatherers, and that the adenosine system then energizes certain endergonic reactions directly, and others indirectly via the GDP, IDP, UDP and CDP systems.

In the study of intact mitochondria[37, 38] it was found that the oxidatively generated ATP could phosphorylate GDP, UDP and CDP but not the corresponding mono-phosphates, although AMP could be readily phosphorylated. The phosphorylation of GMP, UMP and CMP by ATP was brought about by an enzyme found in the soluble fraction of rat liver homogenates[37, 38]. Utter[107] summarizes studies showing that ATP can phosphorylate all of the monophosphates except IMP, and all of the triphosphates including ITP can phosphorylate AMP. ITP alone can phosphorylate IMP by a myokinase-like preparation at least in yeast, and IDP can be converted to ITP by ATP. Deamination of IDP to ADP has been reported in muscle preparations, and dephosphorylation of GDP, IDP and UDP but not ADP or CDP to the monophosphates and inorganic phosphate has been reported in liver and kidney mitochondria[107].

The current picture, though still poorly docu-
mented can at least tentatively be summarized as
shown in Fig. 9.2, which shows the interactions
of the major nucleotides without any attempt to
localize individual reactions in cell compartments,
a maneuver that will ultimately prove very im-
portant but which is beyond the scope of the pres-
ent discussion. In this figure the formation of
GTP, UTP and CTP is shown as one of the func-
tions of the AMP, ADP, ATP system, and the
reaction of these compounds with various phos-
phorylated compounds (XP) yielding GDPX, UDPX
and CDPX to perform various specialized func-
tions was indicated as only one of many ways that
ATP can be used. Before discussing these alter-
native pathways for utilizing ATP it is desirable
to examine the known derivatives of the basic
series that constitute the first 3 in Table 9.1.

B.   *Adenosine mononucleotides of unknown significance*

In the early days of the acid-soluble nucleotides
there were various reports of compounds related
to ATP, and these are represented by Nos. 4, 5
and 6 in Table 9.1. Diadenylic acid, adenylic
dinucleotide or diadenosine diphosphate was re-
ported, as well as the pyrophosphate of this com-
pound[49]. The earlier report was by experts, as
was the much more recent confirmatory report
in 1950[74], in which the compound was reported to
stimulate yeast glycolysis much more than ATP
was able to effect under certain conditions. Fur-
ther studies with snake venom diesterase and
other enzymes, and work with isotopic precursors
seem desirable in order to clear up the signifi-
cance of this compound. Possibly related is No.
6 in Table 9.1, which may be another form of
No. 4, with an additional pyrophosphate. The
structures shown for all 3 compounds are tenta-
tive. The same should probably be said for the

more recently reported tetraphosphate[59] and pentaphosphate[90], Nos. 7 and 8 in the table, and these compounds must also be categorized as being of unknown significance.

FUEL CONSUMPTION: ATP ⟶ SPECIALIZED FUNCTION:

energy-yielding   XP
                                          CTP ⟶ CDPX + PP
reactions utilize
                    P   P   CDP $(PX)_n$ or PXY
inorganic phosphate
                                          CMP ◀
to form phosphate

donors as inter-   ADP   XP
                                          GTP ⟶ GDPX + PP
mediates in glycolysis
                                          GDP ◀⟶ X_n or XY
and oxidation
                                AMP
                                          GMP

                                          UTP ⟶ UDPX + PP   XP

                                          UDP ◀⟶ X_n or XY

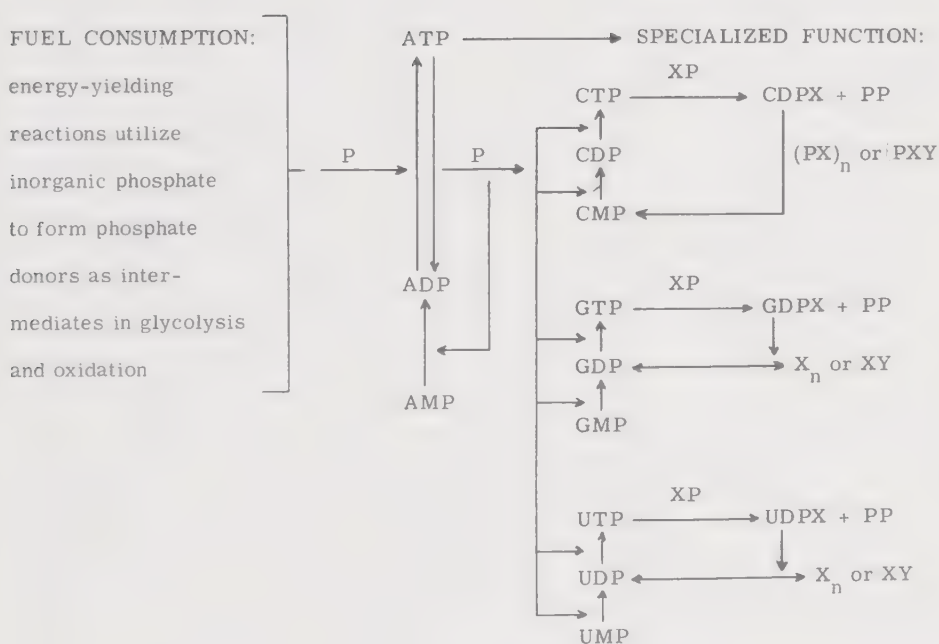   UMP

Fig. 9.2 The adenosine nucleotide system as mediator between fuel consumption and specialized function. The term XP refers to a phosphorylated compound that reacts with a nucleoside triphosphate to form a nucleoside diphosphate X derivative that can regenerate the diphosphate and at the same time insert the X grouping into a polymer (X_n) or a new compound (XY). The specific compounds corresponding to XP, X_n and XY are different for each type of nucleotide. Possibly future research will demonstrate that GTP, UTP, and CTP can react in additional ways that are analogous to the reactions shown by the adenosine nucleotides.

Adenosine-3' phosphate (No. 9) is well known as
a product of RNA breakdown but it has never been
reported as naturally occurring in living tissues.
Its reported occurrence in snake venom[29] might
be fortuitous. However the 3'-triphosphate (No.
10) has been reported in the autotrophic bacterium
Thiobacillus thiooxidans and although the report
has been questioned[36] the occurrence of a series
of structurally related adenosine-3'phosphates as
coenzymes in sulfate metabolism (see below) calls
for a reexamination of the whole question, since
apparently these new compounds have not been
looked for in the sulfur bacteria.

C.   *Adenine mononucleotides with specialized function*

As mentioned above, the report of an adenosine-
3'-triphosphate takes on renewed interest in view
of the series of related compounds that are in-
volved in sulfate metabolism. The simplest of
these is 3', 5'-diphosphoadenosine, No. 11 in the
table, with structure established as indicated.
This compound will catalyse sulfate transfer[33]
with the intermediate formation of 3'-phospho-
adenosine-5'-phosphosulfate[87] (No. 8 in Table
9.2), which can be formed from adenosine-5'-
phosphosulfate[87] (No. 7 in Table 9.2). These
compounds will be discussed later.

Another compound of great interest is No. 12 in
Table 9.1, the internal monophosphate diester 3',
5'-cyclic adenosine monophosphate[105], which was
earlier thought to be cyclodianhydrodiadenylic
acid[36, 105], and has now been identified as the
cofactor for the activation of glycogen phosphoryl-
ase[105]. It was earlier reported to be produced
with ATP and epinephrine or glucagon in a par-
ticulate fraction from a homogenate. More re-
cently it has been shown to accumulate in adrenal

tissue stimulated by the pituitary hormone ACTH, and it was suggested to be a mediator between ACTH and corticosteroid production in the adrenals[35], although the relation between this and the studies by Sutherland and Rall[105] is still not established.

D. *Adenine dinucleotides and other derivatives with specialized function*

The compounds in the previous two sections contained only adenine, ribose and phosphate. In Figure 9.2 it was indicated that type reactions were known by which UTP, CTP and GTP formed diphosphate derivatives which were used in special ways. We shall now list a series of adenine nucleotide derivatives that contain one or more substituents in addition to adenine, ribose and phosphate. Most of these compounds were known before the U-, C-, and GDPX's were discovered, and knowledge of their chemistry and characterization speeded progress on the others.

A fairly complete list of these coenzymes is given in Table 9.2. The first coenzyme to be recognized as some kind of a nucleotide was coenzyme I or diphosphopyridine nucleotide (No. 1 in Table 9.2). It was studied for many years by Euler and by Meyerhof following its discovery in 1904 by Harden and Young, who demonstrated its existence as a cofactor in alcoholic fermentation. Its entire structure [92, 92a] was not known until Warburg and Christian[110] isolated a second coenzyme which they called tri-phosphopyridine nucleotide after showing that it contained three phosphates, with an ADP moiety and a compound that was characterized as nicotinamide attached through a ribose to the terminal phosphate of the ADP moiety (No. 2 in Table 9.2). The chief uncertainty was the position of the extra phosphate in TPN, a point that was established as 2' by Kornberg and

SYNTHESIS AND FUNCTION

Table 9.2   NATURALLY-OCCURRING ADENINE DINUCLEOTIDES AND

SUBSTITUTED MONONUCLEOTIDES

Structure *	Name and Abbreviation, if any	References

1. p-r-N
     |
     p-r-A
	Diphosphopyridine Nucleotide (DPN)	1936 Schlenk and von Euler[92, 92a]

2. p-r-N
     |
     p-r-A
      /
    p (2')
	Triphosphopyridine Nucleotide (TPN)	1935 Warburg et al[110]

3. p-r-F
     |
     p-r-A
	Flavin Adenine Dinucleotide (FAD)	1938 Warburg & Christian[111]
		
		1939 Abraham[1]

4. p-PSH
     |
     p-r-A
      /
     p
	Coenzyme A (CoA)	1954 Lipmann[63]
		
		1954 Wang, Schuster[109]
		
		and Kaplan

5. p-PS-CO-R
     |
     p-r-A
      /
     p
	Acyl Coenzyme A	1951 Lynen et al.[68]

6. CO-R
     |
     p-r-A
	Acyl-AMP	

7. SO_3H
     |
     p-r-A
	Adenosine-5'phosphosulfate (APS)	1958 Robbins & Lipmann[87]

8. SO_3H
     |
     p-r-A
      /
     p
	3'-phosphoadenosine-5'-phosphosulfate (PAPS)	1958 Robbins & Lipmann[87]

9. NH_2
     |
     p-r-A
	Adenosine-5'-phosphoramidate	1958 Katanuma[44]

Table 9.2 continued

10. COO-
      |
p-r-A-CH
      |
     CH$_2$
      |
     COO-
	Succinyladenylate (SAMP)	1956 Carter and Cohen[22]

11. SO$_3$-gluta-
   | myl-serine
 	
p-r-A-
  succinate
		1957 Tsuyuki & Idler[106]

12. p-r-X | pteridine) p-r-A		1958 Moseley and Caputto[72]

*Structural shorthand as in Table 9.1. $\dfrac{p}{\underset{p}{\vert}}$ = same as pp = pyrophosphate;

N = Nicotinamide; F = flavine;

                    r = ribose 2-phosphate;
                     /
                 p (2')

PSH = pantotheine = pantoyl-β-alanyl-mercaptoethanolamine

Pricer[52] and again when the extra phosphate on Coenzyme A was shown to be 3'[109]. The function of DPN and TPN as hydrogen carriers and the definition of a hydrogen carrier as a substance existing in an oxidized and in a reduced form and reduced by an enzyme system separate and distinct from the enzyme system carrying out the oxidation was discussed by Potter[81] in 1941 in a review on the Mechanism of Hydrogen Transport that would be well suited as a preface to modern developments, which undoubtedly run to over a thousand publications[43, 71]. It appears that there are many dehydrogenases that are specific for DPN or for TPN, while some react with both of them and some substrates have separate dehydrogenases that react with DPN and TPN respectively. More and more evidence accumulates to suggest that reduced DPN is oxidized via the cytochrome system by oxygen, generating ATP, while reduced TPN tends to be used for reductive processes in synthetic reactions, although direct transhydrogenation between DPN and TPN seems to be possible[43, 86].
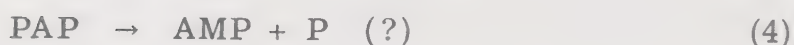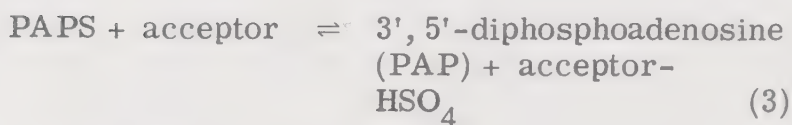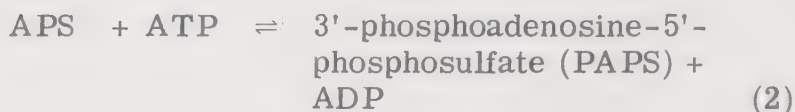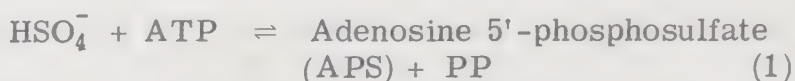
The third nucleotide coenzyme to be discovered was the flavinadenine dinucleotide or FAD (No. 3 in Table 9.2) which was characterized by Warburg and Christian[111] a few years after Theorell showed that riboflavin-5'-phosphate was the prosthetic group of the "old yellow enzyme". A scholarly review of the whole subject of flavin coenzymes has just been published by Beinert[8]. The synthesis of FAD from FMN (flavin mononucleotide or riboflavin-5'-phosphate) is analogous to the syntheses of DPN and TPN and other nucleotide diphosphate-X'syntheses and will be discussed later. Over fifty different flavoproteins have been described[8] and in general these enzymes can be said to function as oxido-reduction catalysts in which the flavin-containing group is tightly bound as a prosthetic group, with very

slight dissociation compared to DPN and TPN. (The distinction between a prosthetic group and a coenzyme is significant mainly in terms of the extent of dissociation.).

Another important coenzyme was discovered by Lipmann who called it coenzyme A because of its acetyl-transferase function[63]. Its structure may be compared with TPN in that an extra phosphate is on the AMP moiety, but curiously, it is on the 3' instead of the 2' position. Its function involves not only acetyl-transferase reactions but acyl transfer in general and some 47 acyl derivatives have either been demonstrated or assumed to occur as part of a series[36]. At least 4 reviews have been written on CoA[36] and the most recent developments in Lynen's laboratory, including terpene synthesis and interaction with biotin-catalyzed reactions will probably be included in a review by Jaenicke and Lynen[42]. These acyl-CoA compounds have the general structure shown in No. 5 in Table 9.2.

Not all of the adenosine nucleotide coenzymes have the nucleotide-diphosphate-X structure, as shown by No. 6 in Table 9.2, which is the general structure of the acyl adenylates[10,46] of which amino-acyl adenylates are a special case[11]. These compounds may be looked upon as mixed anhydrides of the phosphoric acid of AMP with various carboxylic acids.

The corresponding mixed anhydride of AMP with sulfuric acid is shown as adenosine 5'-phosphosulfate or APS (No. 7) which was characterized by Robbins and Lipmann[87]. The activation and transfer of sulfate is brought about by the formation of this compound and its conversion to No. 8 in Table 9.2, the 3'-phosphate of adenosine-5'-phosphosulfate or APS[33, 87]:

$$HSO_4^- + ATP \rightleftharpoons \text{Adenosine 5'-phosphosulfate} \\ \text{(APS)} + PP \tag{1}$$

$$APS + ATP \rightleftharpoons \text{3'-phosphoadenosine-5'-} \\ \text{phosphosulfate (PAPS)} + \\ ADP \tag{2}$$

$$PAPS + \text{acceptor} \rightleftharpoons \text{3', 5'-diphosphoadenosine} \\ \text{(PAP)} + \text{acceptor-} \\ HSO_4 \tag{3}$$

$$PAP \rightarrow AMP + P \quad (?) \tag{4}$$

Reaction 4 is labeled with a question mark because the fate of PAP has not been established in relation to the net uptake of sulfate in reaction 1 and it is conceivable that PAP may be converted to ATP without the intermediary formation of AMP and inorganic phosphate. However, the formation of inorganic phosphate has been a prominent feature of sulfate uptake. The compound PAP was obtained from crude preparations of ADP and ATP[33] and occurs in the acid soluble fraction of certain tissues in very minute amounts. None was found in muscle of any kind and the highest amount was found in liver, which contained only 18 mμmoles per gram[33].

Of the remaining adenine nucleotides, succinyl-adenylate[22] (No. 10) is now well-known as an intermediate in the conversion of IMP to AMP during purine biosynthesis (Chapter VI). The remaining compounds are either incompletely characterized or of unknown function. Tables 9.1 and 9.2 by no means completes the list of reported adenine nucleotides and a number of others are referred to by Henderson and Le Page[36]. An interesting series of compounds related to the adenine nucleotides are those represented by S-adenosyl-methionine which is not a nucleotide but which is formed from ATP[20].

III.   THE URIDINE NUCLEOTIDES

A.   Uridine diphosphate glucose

The long period during which the only acid solu-
ble nucleotides were those containing adenine
lasted from 1914 until 1949, when Luis Leloir
and his colleagues isolated a coenzyme necessary
for the enzymatic conversion of glucose to galac-
tose and showed that it was a uridine ribonucleo-
tide consisting of uridine 5'-diphosphate with a
glucose or a galactose molecule linked to the
terminal phosphate in ester linkage to carbon 1
of the hexose[21]. At about the same time that
UDPG (uridine diphosphate glucose) was isolated,
Park and Johnson detected the accumulation of
urindine-containing substances in bacteria treated
with penicillin and it was subsequently shown that
several compounds containing UDP in combination
with amino sugars and amino acids were pres-
ent[78]. When treated with acid at pH 2.0 for 5
minutes at 100° the hexoses are split off the
above compounds leaving UDP, while with 1N
acid at 100° for 15 minutes the pyrophosphate
is also split yielding 5'-UMP[21]. This compound
was previously unknown, since only the 2'- and
3'- phosphates of uridine had been found in
nucleic acid hydrolysates, but in the following
year Cohn and Volkin obtained 5'-UMP from ribo-
nucleic acid by enzymatic hydrolysis[25]. However
up to the time of these reports the natural occur-
rence of 5'-UMP had not been suspected, despite
the obvious analogies with 5'-AMP.

B.   UMP, UDP and UTP

In their study of the conversion of orotic
acid-6-C[14] into nucleic acid pyrimidines
Hurlbert and Potter[40] observed a large amount
of acid soluble radioactivity that was almost
quantitatively incorporated into RNA and they
undertook its characterization. Early indica-
tions that UMP, UDP and UTP were present

raised the question of the point of attachment of the phosphates, and the work of Leloir[21], of Park[78] and of Cohn and Volkin[25] focussed attention on the possible occurrence of uridine 5'-phosphates analogous to the adenine nucleotides. A compound having the characteristics of a uridine phosphate but shown to differ from uridine 3'-phosphate was reported in 1952 and identified as uridine 5'-phosphate in 1953, when the occurrence of three separate UMP derivatives in the acid soluble fraction were also noted[39], the additional compounds having the phosphate content of UDP or derivatives of it. In the following year the full report showed that the liver from rats injected with orotic acid-6-C^{14} contained radioactive UMP, UDP, UTP, and in addition the UDP-sugars that had been isolated by Leloir[21] and others: UDP-glucose, -galactose, -N-acetyl glucosamine, and -glucuronic acid[40]. During this period the adenine nucleotides were becoming commercially available in quantity and the application of ion-exchange chromatography to crude preparations of yeast nucleotides led to the separation of UTP from ATP[65] and similar work was carried out with ATP from muscle[12]. The work by Hurlbert and Potter[40] established the existence of the uridine nucleotide pool and the close metabolic relationship between the nucleotides vis-a-vis the coenzymes and the nucleic acids, and in collaboration with Schmitz[94] the 5' nucleotides found in RNA were all found in the acid soluble fraction as mono-, di-, and triphosphates. From this it was suggested that the 5'-mononucleotide di- and triphosphates might serve as precursors for both coenzymes and for nucleic acids. Their utilization for nucleic acid synthesis has since become an established fact (Chapter VII) and the conversion of the uridine 5'-phosphates to the uridine coenzymes will be discussed below. Although the deoxy analogue of 5'-UMP (dUMP) is known (Chapter VI), the corresponding diphos-
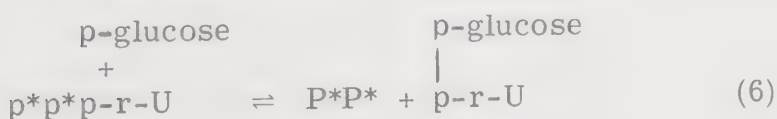
phates and triphosphates have not been demon-
strated in enzymatic reactions, and none of the
three forms has been obtained in the acid soluble
fraction from any kind of cell. This is in contrast
to the cytidine nucleotides (see below) and sug-
gests that dUMP formation is a rate-limiting step
in pyrimidine metabolism.

C. *The formation of uridine diphosphate sugars
from UTP*

According to Leloir[56] the only mechanism known
up to the present for the synthesis of the uridine
diphosphate sugars is the one discovered by
Kornberg[50, 53] for DPN synthesis and generalized
to include derivatives of all of the nucleotides
found in RNA[51]. The enzymes catalysing such
reactions are known by the general name of pyro-
phosphorylases and the specific enzyme for UDPG
synthesis is UDPG-pyrophosphorylase, but it is
also referred to as a pyrophosphate-uridyl trans-
ferase. UDPG is formed by the following rever-
sible reaction:

$$\text{UTP} + \text{glucose-1-phosphate} \rightleftharpoons \text{UDP-glucose} + \text{PP} \qquad (5)$$

This reaction can be shown schematically using
the structural shorthand of Table 9.1 as follows,
with the pyrophosphate P shown as p*p* in UTP:

$$
\begin{array}{lll}
\text{p-glucose} & & \text{p-glucose} \\
+ & & | \\
\text{p*p*p-r-U} & \rightleftharpoons \text{P*P*} + & \text{p-r-U}
\end{array}
\qquad (6)
$$

These reactions represent the synthesis of
UDP-X compounds from UTP as shown in Fig.
9.1, where X-P is a sugar 1-phosphate.

The following compounds have been shown to oc-
cur in combination with UDP in forms analogous
to UDPG and are believed to be formed by reac-
tions analogous to reaction 5 and Fig. 9.2:

XP		UDP-X
glucose-1-P	$\rightleftharpoons$	UDP-glucose
		$\updownarrow$
galactose-1-P	$\rightleftharpoons$	UDP-galactose
N-acetyl-glucosamine-1-P	$\rightleftharpoons$	UDP-acetyl glucosamine
		$\updownarrow$
N-acetyl-galactosamine-1-P	$\rightleftharpoons$	UDP-acetyl galactosamine
L-arabinose-1-P	$\rightleftharpoons$	UDP-arabinose
		$\updownarrow$
D-xylose-1-P	$\rightleftharpoons$	UDP-xylose

In the above list it is to be noted that interconversions at the UDP-X level occur as isomerizations. UDP-acetyl glucosamine can also be converted to the mannose derivative. These reactions are brought about by inversion of the hydroxyl at C-4 of the hexoses and have been shown to require DPN, presumably with the intermediate formation of a 4-keto aldose. In addition to the interconversions shown, many of the UDP-X compounds can be converted to other UDP derivatives, thus UDPG can be oxidized to UDP-glucuronic acid in the presence of DPN and this can be converted to UDP-galacturonic acid. All of these UDP-X compounds are utilized as shown in Fig. 9.1 with the formation of polymers of X (X_n) or derivatives of X (XY), and the regeneration of UDP. Some examples will be given below. At the time of this writing there are no confirmed reports of uridine nucleotides occurring naturally other than UMP, UDP, UTP and compounds that consist of UDP with additional groupings on the terminal phosphate, i.e. members of the UDP-X series.

D.   *Utilization of UDP-sugars*

The UDP-X compounds can be utilized for the synthesis of many carbohydrates and carbohydrate

derivatives including sucrose, lactose, cellulose, glycogen, trehalose phosphate, glucuronides, chitin, hyaluronic acid, and bacterial cell wall components. The series of compounds isolated by Park include a UDP-acetyl-glucosamine with carbon 3 of the hexose linked to position 2 of lactic acid through an ether and is thus a UDP-acetyl-muramic acid[56], which is his compound 1. Compound 2 has alanine in peptide linkage with the carboxyl of the lactic acid, and compound 3 has additional amino acids in peptide linkage with the alanine. These compounds are probably intermediates in the formation of bacterial cell walls.

In addition to the isomerizations mentioned above, the various products of glycosyl group transfers have been categorized by Leloir[56] as follows:

Disaccharides, e.g. sucrose, lactose.

β-glucuronides, e.g. with aminophenols, tetra-hydrocortisone, phenolphthalein, thyroxine.

Glucosides, e.g. with anthranylic acid.

Galactolipids, e.g. with rat brain microsome preparations.

Polysaccharides, e.g. cellulose and glycogen.

The latter example is of great interest because liver glycogen had been assumed to be formed by the reversal of its breakdown by phosphorylase. However Leloir and Cardini[55] discovered that UDPG could serve as a source of glycogen. Further work has been carried out by Robbins, Traut and Lipmann[88] who pointed out that synthesis of glycogen from UDPG should have a standard free energy change of about -3000 calories per glucose unit so that conversion should be

greater than 99%. It appears that glycogen synthesis may take this pathway while phosphorylase may function mainly for glycogen breakdown.

The available data suggest that the uridine nucleotide coenzymes represent a specialized category of metabolic reactions and that all of their activity centers around carbohydrate derivatives and their polymerization or condensation. While no immediate reason for this is apparent it will be interesting to note whether the other nucleotide coenzymes are similarly specialized, and whether future research on the uridine nucleotides will be as specialized as it has been up to now.

IV.   *THE CYTIDINE NUCLEOTIDES*[5,36,47]
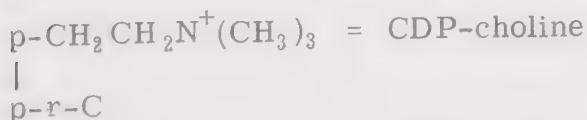
A.   *CMP, CDP and CTP*

The work of Cohn and Volkin[25] showed that 5'-CMP could be obtained from RNA by treatment with snake venom diesterase, but no acid soluble cytidine nucleotides were known until the work of Schmitz et al[94, 95] which was carried out in parallel with the work of Hurlbert and Potter[40]. The first isolation of CTP was carried out with extracts from Flexner-Jobling carcinoma tissue from animals injected with radioactive glucose[95] and this material was used for chemical characterization[94]. In the studies with labeled orotic acid, the cytidine nucleotides were labeled to about the same extent as the uridine nucleotides[40]. During these investigations no evidence for deoxyribonucleotides was obtained, but later work by R. L. Potter et al[84] showed that the acid soluble fraction from calf thymus tissue contained dCMP, dCDP and dCTP in amounts equivalent to about 2/3 of the total cytidine 5' phosphates. In addition, evidence was obtained for the existence of two new deoxycytidine diphosphate compounds one of which was
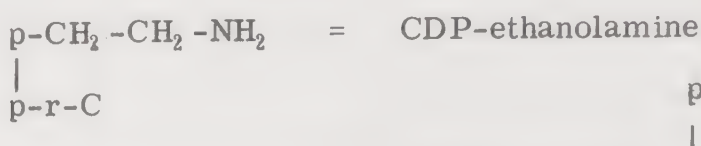
later identified as dCDP-ethanolamine[83] which was accompanied by CDP-ethanolamine (see below).   No deoxyuridine nucleotides were found but the (deoxy) thymidine nucleotides were obtained as TMP, TDP and TTP in calf thymus[84].

B.   CDP-X Compounds

Kennedy and Weiss[48] discovered CDP-choline and CDP-ethanolamine as essential intermediates in the biosynthesis of the phospholipids during the course of a study of the enzymic incorporation of phosphorylcholine into lecithin.   The reaction was stimulated by amorphous preparations of ATP but not by crystalline preparations, and the results were traced to the presence of CTP in the amorphous ATP.   Apparently making the assumption that CDP-choline and CDP-ethanolamine were the intermediates, these compounds were synthesized by the methods of Khorana using radioactive precursors.   The radioactive CDP derivatives were added to extracts of rat or chicken liver and chromatographed by the gradient elution method.   The radioactive peaks were located, purified, and found to be diluted from 10 to 40 fold.   From the dilution it was calculated that rat liver contained 5 and 10 μ moles of CDP-ethanolamine and CDP-choline respectively per 100 grams fresh weight while chicken liver contained about 6X as much of each.   R. L. Potter et al found CDP-ethanolamine and dCDP-ethanolamine in the acid soluble fraction of thymus tissue[83].   Sugino[104] has found dCDP-choline in sea urchin eggs and this may be one of the unidentified compounds in thymus[83].
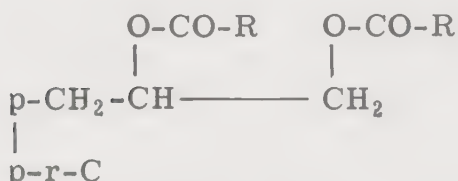
The structure of the above compounds corresponds to the UDP-X series, in which the terminal P is esterified with an ROH. They can be shown in the notation of Fig. 9.2 as follows:

$$p\text{-}CH_2CH_2N^+(CH_3)_3 \ = \ CDP\text{-choline}$$
$$|$$
$$p\text{-}r\text{-}C$$

$$p\text{-}CH_2\text{-}CH_2\text{-}NH_2 \quad = \quad CDP\text{-ethanolamine}$$
$$|$$
$$p\text{-}r\text{-}C$$

$$p$$
$$|$$

Analogous structures for dCDP would show p-dr-C.

More recently, Paulus and Kennedy[7] have chemically synthesized two types of CDP-diglycerides, the -dipalmitin and the -dilaurin and have presented evidence for their synthesis and function. The structures are

$$\begin{array}{cc} O\text{-}CO\text{-}R & O\text{-}CO\text{-}R \\ | & | \\ p\text{-}CH_2\text{-}CH\text{------} & CH_2 \\ | & \\ p\text{-}r\text{-}C & \end{array}$$

In addition to the above compounds, Baddiley et al[6] isolated two more CDP-X compounds from <u>Lactobacillus</u> <u>arabinosus</u>, in which X is ribitol and glycerol, respectively.

C.   *Synthesis of CDP-X Compounds*

The synthesis of the CDP-X compounds parallels that of the UDP-X compounds as shown in Fig. 9.2 and may be looked upon as a pyrophosphorylase type[14].

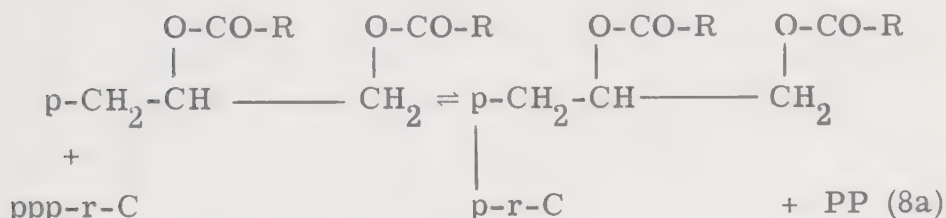$$CTP + p\text{-choline} \rightleftharpoons CDP\text{-choline} + PP. \qquad (7)$$

The enzyme catalysing the above reaction is also referred to as phosphoryl-cytidyl transferase. The enzyme forming CDP-ethanolamine is analogous in mechanism[47] but appears to be soluble while the choline enzyme is in the particulate fraction of cytoplasm[14].

In the synthesis of the CDP-diglycerides[3] a phosphatidic acid reacts with CTP as follows:

CTP + phosphatidic acid $\rightleftharpoons$ CDP-diglyceride + PP   (8)

Using the shorthand system of Fig. 9.2 this would be shown in the following manner.

$$
\begin{array}{ccc}
\text{O-CO-R} & \text{O-CO-R} & \text{O-CO-R} \quad \text{O-CO-R} \\
| & | & | \qquad\qquad | \\
\text{p-CH}_2\text{-CH} \text{———— CH}_2 & \rightleftharpoons \text{p-CH}_2\text{-CH} & \text{———— CH}_2 \\
+ & & | \\
\text{ppp-r-C} & \text{p-r-C} & + \text{ PP (8a)}
\end{array}
$$

Similar reactions appear to occur between CTP and D-ribitol-5-phosphate and L-α-glycerophosphate in L. arabinosus extracts to form CDP-ribitol and CDP-glycerol respectively[97]. All of these reactions are apparently analogous to the reactions generating UDP-X.

D. *Function of CDP-X compounds*

The function of the CDP-X compounds is to serve as donors of activated X groupings for polymerizations or condensations with other compounds to form new substances as in Fig. 9.2. Specific examples can be given for each of the CDP-X compounds. The reactions appear to differ from the UDP-X series in that CMP is formed from CDP-X and the terminal phosphate of CDP accompanies the X, whereas UDP is formed from the UDP-X in the process of group transfer and the terminal phosphate remains on UDP.

1. Phospholipid synthesis. Kennedy and his coworkers have shown that the enzymic synthesis of phospholipids involves the cytidine coenzymes[47]. The following reaction is catalyzed by phosphorylcholine - glyceride transferase[112]:

CDP-choline + D-α, β –diglyceride $\rightleftharpoons$ lecithin + CMP   (9)

Particulate preparations from rat liver and chicken liver were used. Magnesium or manganese ions activated the system while calcium ions were inhibitory. The enzyme is also involved in lecithin synthesis in brain and nerve tissue.

The following reaction is catalyzed by phosphoryl-ethanolamine-glyceride transferase[48]:
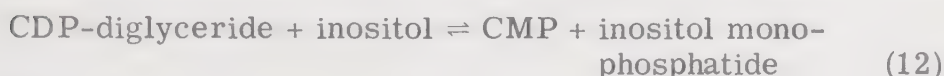
$$\text{CDP-ethanolamine} + \text{D-}\alpha, \beta\text{ -diglyceride} \rightleftharpoons \text{phosphatidylethanolamine} + \text{CMP} \quad (10)$$
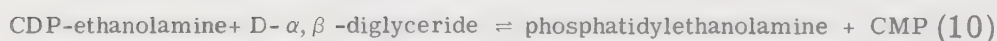
The following reaction is catalyzed by phosphorylcholineceramide transferase[99]:

$$\text{CDP-choline} + \text{N-acyl-D-}\underline{\text{threo}}\text{-}\underline{\text{trans}}\text{-sphingosine} \rightleftharpoons \text{sphingomyelin} + \text{CMP} \quad (11)$$
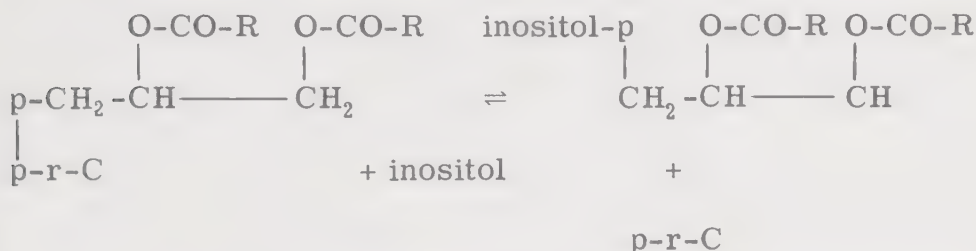
The enzyme was demonstrated in chicken liver particulate fractions.
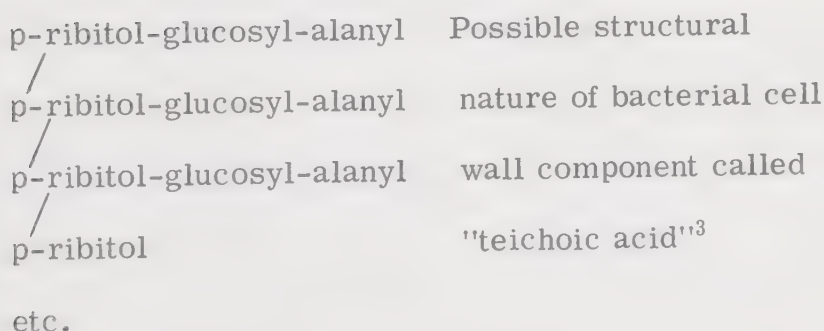
2. Synthesis of inositol monophosphatide. In 1958 Agranoff et al.[2] reported the synthesis of inositol monophosphatide in a system containing inositol, phosphatidic acid and CDP-choline or CMP and proposed a reaction sequence in which CDP-choline reacted with the phosphatidic acid to form CDP-diglyceride which then reacted with inositol to form inositol monophosphatide plus CMP. In 1960 Paulus and Kennedy[79] modified this scheme, rejecting CDP-choline as an intermediate and proposing the direct formation of CDP-diglyceride in the now classic pattern, starting with CTP (see above). The remainder of the scheme was in agreement with the earlier proposal:

CDP-diglyceride + inositol $\rightleftharpoons$ CMP + inositol mono-
phosphatide (12)

or,

$$
\begin{array}{ccccccc}
\text{O-CO-R} & \text{O-CO-R} & & \text{inositol-p} & \text{O-CO-R} & \text{O-CO-R} \\
| & | & & | & | & | \\
\text{p-CH}_2\text{-CH}\!-\!\!-\!\!-\!\!-\text{CH}_2 & & \rightleftharpoons & \text{CH}_2\text{-CH}\!-\!\!-\!\!-\text{CH} \\
| & & & & \\
\text{p-r-C} & & +\ \text{inositol} & & + \\
& & & & \text{p-r-C}
\end{array}
$$

3. <u>Ribitolphosphate and glycerophosphate polymers</u>. The isolation of CDP-ribitol and CDP-glycerol was referred to earlier[6]. The possible significance of these compounds is indicated in reports from the same laboratory[3,7] reporting a substance giving the appearance of either a mixed polymer of ribitol phosphate and glycerophosphate or a mixture of polymers of each, although the latter seemed preferred[3]. The amount of phosphate in the polymer suggested that the monomers were linked by the phosphate of one to a hydroxyl of the other somewhat as follows,

p-ribitol-glucosyl-alanyl   Possible structural
/
p-ribitol-glucosyl-alanyl   nature of bacterial cell
/
p-ribitol-glucosyl-alanyl   wall component called
/
p-ribitol   "teichoic acid"[3]

etc.

in a structure resembling the backbone of a nucleic acid, and having phosphates in diester linkage, without specifying the hydroxyls involved. The polyribitol also contained glu-

cose and alanine in bound form. It was shown that the alanine was all in ester linkage with a hydroxyl of the glucose or of the ribitol, and the ratio of glucose to alanine was 1:1 suggesting that they were associated. However it was not clear what percentage of ribitol moieties carried glucose and alanine[3]. The occurrence of phosphate in the product suggests that the transfer function removes the terminal phosphate from CDP-ribitol or -glycerol yielding CMP as in the earlier examples. Although the compound was obtained from L. arabinosus, Kennedy[47] points out that cardiolipin, a substance isolated from beef heart, is probably a polyglycerophosphate esterified with long chain fatty acids and he points out the analogy between this and the polymers obtained by Baddiley.

4. Conversion of Ribotides to Deoxyribotides. Reichard and Rutberg[85] have recently reported that CDP could be converted to dCDP in the presence of reduced TPN and an enzyme from E. coli and reserved the decision as to whether the reduction involves CDP or a derivative of it, although CMP and CTP seemed to be excluded. Kennedy[47] has speculated as to the significance of the existence of dCDP-choline and dCDP-ethanolamine as well as CDP-choline and CDP-ethanolamine and has raised the question of whether CDP-choline or -ethanolamine is reduced to the corresponding dCDP derivative. Perhaps a suitable test system would be rat thymus, where all 4 compounds apparently are found as in calf thymus[83] and where DNA is being synthesized.

5. Synthesis of Pantotheine. Brown[16] has reported that CTP is required for one of the

steps in CoA synthesis, the condensation of 4'-phosphopantothenic acid with cysteine, and has suggested the occurrence of the following reaction:

4' phosphopantothenic acid + cysteine + CTP →
    4' phosphopantothenylcysteine + CDP + P   (13)
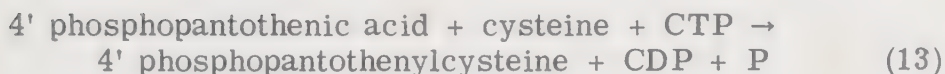
This reaction was specific for CTP in extracts of E. coli and Proteus morganii but was satisfied by GTP, UTP and ATP as well, when extracts of rat liver were used. The reaction was written as above in the preliminary communication with the reservation that the identification of the products CDP and P was only tentative and later[16] it was again stated that the formation of CMP and PP was not ruled out. However it was reiterated that no compound containing both cytosine and sulfur (i.e. no CDP-X) could be demonstrated. The issue is here emphasized because if CDP and P are formed it would be the first departure from the type reaction shown for the CTP utilizations as shown in Fig. 9.2, and would indicate a trend in the direction taken by the adenine nucleotides (see below) i.e. multiple mechanisms, and Brown implies a one-enzyme mechanism for reaction 13. The report by Brown is of great interest in that it indicates 2 possible routes for CoA synthesis, one previously considered and now shown to be inadequately supported. The issue of de novo versus preformed pathways in this system does not appear to have been discussed.

V. THE GUANOSINE AND INOSINE NUCLEOTIDES[107]

Guanosine and inosine nucleotides will be discussed together since there are only a few examples of enzymatic reactions requiring these nucleotides, and it

appears that in several instances the nucleotides re-
place each other[107].

A.   Natural occurrence

The first report of GTP was based on its occur-
rence as a contaminant in ATP prepared from
rabbit muscle[12].   The compound was soon shown
to occur in several tissues[107] and was nicely dem-
onstrated by means of rechromatography of com-
bined GTP and UTP fractions, viz. Fig. 2 by
Schmitz et al[94].   This figure is also of interest
because it provides no evidence for the occurrence
of ITP (see below).   Schmitz et al also found GMP
and GDP in the acid soluble fraction of a rat car-
cinoma, viz. Chart la, [95] and other tissues.

The question of the natural occurrence of ITP and
IDP is in need of reinvestigation.   Utter[107] refers
to a report by Siekevitz and Potter[98] as one of the
few indications for ITP in tissues.   This paper
was not concerned with the existence or non-
existence of ITP but was concerned with the me-
tabolism of ATP in rat liver mitochondria, and a
fraction eluted after ATP was labeled ITP (Fig.
1, Paper II)[98] on the basis of its radioactivity
in relation to ATP, its spectrum, and position of
a known standard.   A paper by Brumm et al[17] re-
ported work done at about the same period, in
which the acid soluble fraction of rat liver was
examined 15 min. after the injection of 100 μc
of inorganic phosphate. A large peak of radio-
activity was found in the GTP-UTP position and
was obviously not in either of these 2 compounds.
ITP would elute between GTP and UTP on this
chromatogram in which IMP elutes between GMP
and UMP, but as mentioned in an example above,
no rechromatogram showing ITP resolved from
a natural mixture of GTP and UTP has been pub-
lished, and none of the data from our laboratory
can be regarded as proof of its natural occur-

rence.   An unknown nucleotide in the acid soluble fraction of whole blood from leukemic patients[113] was found in the GTP-ITP-UTP position, and remains a candidate for identification as ITP on the slender basis of the fact that almost no other nucleotides besides the adenine type were found. The issue is not whether IMP occurs naturally, for it surely does, or whether there are enzymes that can convert IMP to IDP and ITP, for these do occur.   The main question is whether IMP is converted to other products such as hypoxanthine, succinyl-AMP, and xanthylic acid (XMP) so rapidly that in most situations there is not enough IMP available for IDP or ITP synthesis, or whether these compounds are rapidly converted to other forms.   The non-occurrence of IMP in nucleic acids suggests that IDP and ITP must have very limited distribution in tissues.   It may be noted at this point that XMP occurs only in very small quantities yet it is an important intermediate in purine biosynthesis (Chapter VI) without having been found as either the diphosphate or triphosphate or in nucleic acids.

A GDP-X compound, in which X was identified as mannose, was found in yeast by Cabib and Leloir in 1954[19].

No reports of the occurrence of the deoxyriboside 5'-phosphates or polyphosphates of guanine or hypoxanthine in detectable amounts in the acid soluble fraction of cells have appeared.   The formation of deoxy-GTP and its incorporation into DNA is of course well established (Chapter VII).

B.   *Synthesis and function*

The formation of IMP, XMP, and GMP has been discussed in Chapter VI on purine biosynthesis.   Here we are concerned with the formation of the higher phosphates and

nucleoside diphosphate-X compounds if they occur. Transphosphorylases for exchanging the pyrophosphate phosphorus of the various di- and triphosphates are readily demonstrated in a variety of tissues[107] with several suggestions of specificity. Herbert and Potter[37] suggested that GMP and GDP are phosphorylated by ATP and are not primary acceptors from the reactions of oxidative phosphorylation. However GDP appears to be required as a phosphate acceptor and is not replaced by ADP in the reaction:

$$\text{Succinyl-S-CoA} + \text{GDP} + \text{P} \rightleftharpoons$$
$$\text{Succinate} + \text{CoA-SH} + \text{GTP} \qquad (14)$$
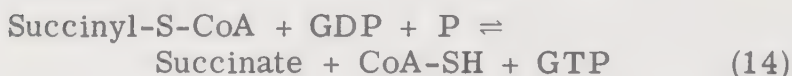
as shown by Sanadi et al[91]. IDP was nearly as effective as GDP, while ADP, CDP and UTP were without activity. A similar relationship between the five nucleotides was found in the case of phosphopyruvate carboxykinase[54]:

$$CO_2 + \text{GDP} + \text{phosphopyruvate} \rightleftharpoons \text{oxalacetate} + \text{GTP} \qquad (15)$$
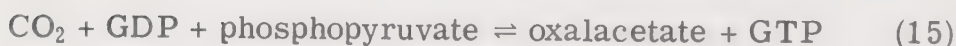
It seems likely that the reverse of this reaction is important in the process of gluconeogenesis from protein and that it is the same reaction observed by Kalckar in 1939.

It is completely unknown at this time whether the guanosine or the inosine polyphosphates are the physiological cofactors in the above two reactions, since unequivocal studies on the above two reactions do not appear to have been carried out in terms of endogenous mitochondrial nucleotides, but this should be possible because the enzymes are mitochondrial in origin. The high degree of specificity for GDP and IDP in these 2 reactions may be limited to certain animal tissues since Utter[107] cites data on plant and bacterial systems which were specific for ATP.

In the above reactions it would seem difficult to say whether we are dealing with GTP synthesis or GTP function, but it would appear that most of the time GTP is synthesized from GDP by ATP through the action of nucleoside diphosphokinase and that the above reactions represent GTP utilization.

Additional functions for GTP include the synthesis of AMP from IMP via succinyl-AMP[60].

$$IMP + \text{L-aspartate} + GTP \rightleftharpoons S\text{-}AMP + GDP + P \quad (16)$$

$$S\text{-}AMP \rightleftharpoons AMP + \text{fumarate} \quad (17)$$

In this reaction it was found that ITP showed less than 1% as much activity as GTP.

Another reaction that appears to involve GTP preferentially is the incorporation of amino acids into microsomal protein, in which ITP is 10% as effective and the other triphosphates are ineffective[45]. The role of GTP is not clear but it appears to involve the transfer of amino acids in general from transfer RNA to microsomal protein (Chapter XI).

There is only one clear example of a GDP-X utilization of GTP and this is the formation of GDP-mannose[73]:

$$GTP + \text{mannose-1-P} \rightleftharpoons GDP\text{-mannose} + PP \quad (18)$$

which is a specific example of the type reaction shown in Fig. 9.2. As of this writing there are no examples of an IDP-X demonstrated to occur naturally or in an enzyme system.

VI. THE THYMIDINE NUCLEOTIDES

A. TMP, TDP and TTP

The thymidine nucleotides were discovered in the acid soluble fraction of thymus tissue by R. L. Potter et al[84]. The corresponding ribose derivative has been found in RNA but there are no reports of the occurrence of thymine riboside phosphates in the acid soluble fraction, although these compounds presumably occur. Studies on the conversion of TMP to TTP have been carried out with ATP as the phosphorylating system (Chapter VII) but other phosphate donors have not been studied. The thymidine 5'-phosphates have also been reported in the acid soluble fraction of E. coli[76].

B. The TDP-X Compounds

It was in the course of the search for TMP, TDP and TTP in E. coli that Okazaki et al[76] discovered evidence for the occurrence of two additional thymidine derivatives which were referred to as TDP-X_1 and TDP-X_2. The total radioactivity of the 5 compounds passed rather quickly into the DNA fraction, but this does not prove that any compound other than TTP was a primary precursor since the TDP-X compounds might be in rapid equilibrium with the other forms. The identification of TDP-X as TDP-rhamnose (6-deoxymannose) was reported by Okazaki[75] in 1959. This was the first of the TDP-X compounds. The occurrence of this class of compounds was quickly confirmed by other investigators who were experienced in the chemistry of the UDP-X and CDP-X compounds. Strominger and Scott[102] reported three TDP-X compounds in E. coli and indicated some of the difficulties that prevented them from making positive identification of the X-moieties which appeared to be unusual sugars. Baddiley and Blumson[4] studied Streptomyces griseus and obtained 2 compounds which were identified as TDP-mannose and TDP-rhamnose. They suggested that

since the organism produces Streptomycin B
which is an α-D-mannopyranosyl-streptomycin,
the compounds might function in its synthesis.

## VII. THE MULTIPLICITY OF ADENINE NUCLEOTIDE REACTIONS

Several neat generalizations can be made about the
nucleotides that contain bases other than adenine: they
appear to function in group transfer by forming a
nucleoside-diphosphate-X group from a nucleoside
triphosphate and a phospho-X, splitting out pyrophos-
phate (Fig. 9.2). The CDP-X compounds appear to
transfer X with P attached, leaving CMP to recycle
through CDP, CTP, and CDP-X. The UDP-X com-
pounds appear to transfer X without attached P, re-
generating UDP instead of UMP. The single example
of GDP-X is formed in the UDP-X manner and prob-
ably functions by transferring X but not P, though
this is not yet demonstrated. The function of TDP-X's
is still unknown.

Perhaps these apparently simple rules are merely the
result of the fact that biochemists have only been work-
ing with these compounds since about 1955 and methods
and ideas very readily become stereotyped. Perhaps
when as many experiments have been done with the
newer nucleotides they will show the versatility of the
adenine compounds. In any case we can only marvel
at the range of activities shown by the adenine nucleo-
tides - we cannot generalize or predict whether they
will always be as dominant as they now appear to be
or whether there will ever be an end to the growing
list of enzyme mechanisms involving the adenine
nucleotides. A brief survey of some of these reactions
may suggest what lies ahead for students of the other
nucleotides, and at the least it should help to dispel
the comfortable idea that nature is simple or that
"nature prefers the simplest way" or that when one
pathway is known no others need be expected. There
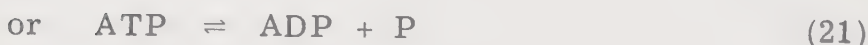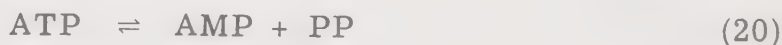appears to be a multiplicity of pathways by which ATP

can be used and in an increasing number of instances
there is more than one pathway leading to the same
end product.

At the present time we can only attempt a beginning
classification of ATP-utilizing mechanisms, and indi-
cate some of the sources of further information. The
review by Bock[12] lists a number of relevant discus-
sions of the problem and in addition it is a good pri-
mary source.

The basic problem under discussion is the "synthase"
problem, i. e. how can the energy of ATP or any other
nucleoside triphosphate by utilized for attaching a com-
pound X to a compound Y to make the compound XY, in
those instances where the reaction is endergonic
(energy-requiring)?

$$X + Y + energy \ \rightleftharpoons \ XY \tag{19}$$
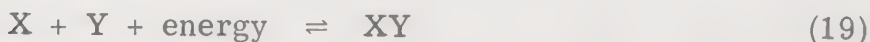
This reaction may be coupled with either one of the
following reactions:

$$ATP \ \rightleftharpoons \ AMP + PP \tag{20}$$

$$or \quad ATP \ \rightleftharpoons \ ADP + P \tag{21}$$

Thus the overall reactions are

$$\boxed{Type\ 1:} \ ATP + X + Y \rightleftharpoons XY + AMP + PP \tag{22}$$

$$\boxed{Type\ 2:} \ ATP + X + Y \rightleftharpoons XY + ADP + P \tag{23}$$

Lipmann[64] has referred to these as the Type 1 and
Type 2 mechanisms respectively, and regarded the
reactions as displacements of pyrophosphate by X in
the Type 1 and displacement of ADP by X in the Type
2. In the Type 1 reaction the pyrophosphate bond be-
tween AMP and PP is broken and in the Type 2 reac-
tion the pyrophosphate bond between ADP and P is
broken, so that we may characterize the two types

conveniently in terms of "pyrophosphate-release" or "phosphate-release". These two mechanisms can be further subdivided according to whether the phosphate or pyrophosphate release is brought about by a single enzyme or by the successive action of two or more enzymes. The four categories of ATP or other nucleoside triphosphate utilization that we shall employ are thus the following:

Type 1 (a) Two-enzyme pyrophosphate-release (Kornberg).
(b) One-enzyme pyrophosphate-release (Berg, Hoagland).

Type 2 (a) Two enzyme phosphate-release (Cori, Lipmann).
(b) One enzyme phosphate-release (Buchanan, Hartman).

Historically the "two-enzyme phosphate-release" was the first to be known and understood, and it took a number of years to appreciate the fact that this was not the only way to carry out "synthase" reactions. The various types will now be discussed with some attempt at historical perspective.
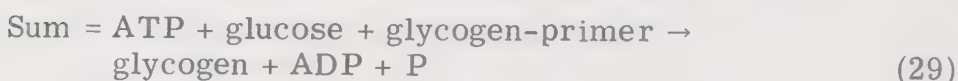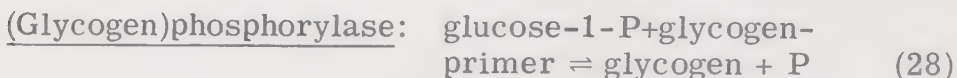
A.   *Two-enzyme phosphate-release*

In this mechanism, reaction 23 can be shown to be the sum of two reactions, each of which is catalysed by a different enzyme:

Phosphokinase: $ATP + X \rightleftharpoons XP + ADP$ (24)

Phosphorylase: $XP + Y \rightleftharpoons XY + P$ (25)

The sum of reactions 24 and 25 is reaction 23. The classical type of ATP utilization by two-enzyme phosphate-release was described by Lipmann in 1941 and in 1946[61]. A typical example was glycogen synthesis which was demonstrated

to take place in the presence of glucose and ATP and the appropriate enzymes in a <u>tour de force</u> by Colowick and Sutherland in 1942[26].

<u>Hexokinase</u>:   ATP + glucose → glucose-6-P + ADP   (26)

<u>Phosphoglucomutase</u>: glucose-6-P ⇌ glucose 1-P   (27)
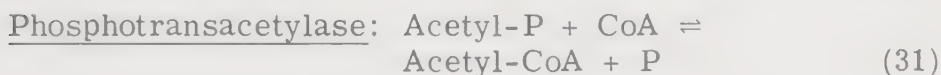
<u>(Glycogen)phosphorylase</u>:   glucose-1-P+glycogen-
                                   primer ⇌ glycogen + P   (28)

Sum = ATP + glucose + glycogen-primer →
      glycogen + ADP + P   (29)
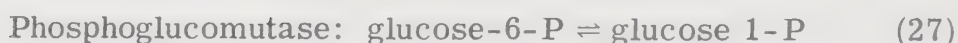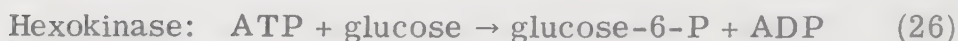
The first step is catalysed by <u>hexokinase</u> and leads to the formation of the XP and ADP.   The second step is a rearrangement catalysed by <u>phosphoglucomutase</u>.   The third step is catalyzed by a different enzyme, <u>phosphorylase</u>, forming X_n + inorganic phosphate.   In this example it should be pointed out that Y is represented by glycogen-primer in equation 29 which should be compared with equation 23.   This basic scheme served as a model for all of the thinking on the subject during this period, as is clearly shown by Lipmann's representation of fat and protein synthesis from phosphorylated amino acids and fatty acids[61] and as shown in Fig. 1 of the review by Potter[82].

The next example of the two-enzyme phosphate-release type is represented by acetate transfer. The early work by Lipmann[61, 62] showed that certain bacteria can generate acetyl-phosphate in the course of pyruvate breakdown and also demonstrated that in these bacteria acetate could be activated by ATP in the <u>acetokinase</u> reaction, which was later studied in detail in Ochoa's laboratory[89]:
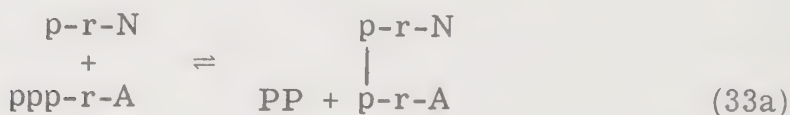
Acetokinase: ATP + acetate $\rightleftharpoons$ Acetyl-P + ADP   (30)

Phosphotransacetylase: Acetyl-P + CoA $\rightleftharpoons$
Acetyl-CoA + P   (31)

Sum   ATP + Acetate + CoA $\rightleftharpoons$ Acetyl CoA +
ADP + P   (32)

The second step in acetate activation in these organisms was brought about by phosphotransacetylase, which was studied in detail by Stadtman[100]. The above pair of reactions represent formation of an XP and ADP, followed by reaction with Y to form XY and inorganic phosphate in the presence of a second enzyme. It may be noted here that the second enzyme might have been called "Acetyl-CoA phosphorylase" according to present day nomenclature. All attempts to find this enzyme in animal tissues failed, although acetyl CoA could react with various acetyl acceptor systems in animal tissues[23]. The search for the enzymatic equivalent of phosphotransacetylase in animal tissues culminated in an excellent series of papers by Berg, who also reviewed the prior literature[110]. These studies revealed that a new mechanism for the formation of acetyl-CoA occurred in animal tissues, yeast, plants and at least one species of bacteria. It differed in two important ways from the system found in the bacteria studied earlier. Instead of forming ADP and inorganic phosphate, it formed AMP and pyrophosphate as end products, and instead of two enzymes it appeared to require only one. Historically, it was preceded by the work of Kornberg[50] which will be described in the next section.

B. *Pyrophosphate release*

In 1950 Kornberg[50] reported the synthesis of DPN from nicotinamide mononucleotide (NMN) and ATP in a reaction that yielded pyrophosphate:

DPN pyrophosphorylase: NMN + ATP $\rightleftharpoons$ DPN + PP   (33)

The mechanism of the reaction can be presented in the notation of Table 0.1 as follows:

$$p\text{-}r\text{-}N \qquad\qquad p\text{-}r\text{-}N$$
$$+ \qquad \rightleftharpoons \qquad\qquad |$$
$$ppp\text{-}r\text{-}A \qquad\quad PP + p\text{-}r\text{-}A \qquad\qquad (33a)$$

In discussing this reaction Kornberg pointed out that "the known reactions of ATP involve either a hydrolytic cleavage of one of the pyrophosphate linkages or a transfer of the terminal phosphate group to an acceptor molecule such as creatine, adenylic acid, or a sugar. The condensation of ATP with a mononucleotide to split out PP and form a dinucleotide has not been observed previously. The reverse reaction, namely the cleavage of a dinucleotide by PP, resembles the phosphorolytic splitting of glycogen, disaccharides, and nucleosides and by analogy may be termed "pyrophosphorolytic"[50]. The model was shown to apply to the synthesis of FAD as well, and also proved to be the mechanism for synthesizing the UDP-X, CDP-X and GDP-X compounds as described earlier.[51]

C.   *Two-enzyme pyrophosphate-release*

Following the discovery of the first pyrophosphorylase, which represented a rather special type of ATP utilization because both the energy and the AMP residue were utilized, additional reactions were found. A model which is analogous to the two-enzyme phosphate release is as follows:

Pyrophosphokinase: ATP + X $\rightleftharpoons$ X-PP + AMP   (34)

Pyrophosphorylase: X-PP + Y $\rightleftharpoons$ XY + PP   (35)
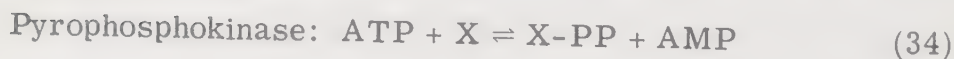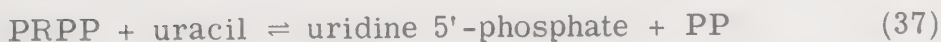
The sum of reactions 34 and 35 is reaction 22. An example of reaction 34 is the formation of

phosphoribosyl pyrophosphate (PRPP) in which ribose-5-phosphate replaces X:

$$ATP + ribose\ 5\text{-}phosphate + PRPP + AMP \qquad (36)$$

An example of reaction 35 is the synthesis of uridylic acid in which uracil replaces Y in reaction 35:

$$PRPP + uracil \rightleftharpoons uridine\ 5'\text{-}phosphate + PP \qquad (37)$$

The sum of reactions 34 and 35 is:

$$ATP + ribose\ 5\text{-}phosphate + uracil \rightleftharpoons$$
$$uridine\ 5'phosphate + AMP + PP \qquad (38)$$
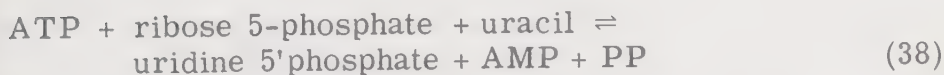
Reaction 38 is analogous to reaction 22. Kornberg[51] reported that not only uracil but also adenine, guanine, hypoxanthine and orotic acid participate in reactions analogous to reaction[37], forming the corresponding ribotides, and subsequently many purine and pyrimidine analogs have been shown to react in this way. However it was soon to be found that pyrophosphate release could be effected by what has so far appeared to be a two-step reaction catalysed by a single enzyme.

D.   *One-enzyme pyrophosphate release*

The details of the one-enzyme pyrophosphate release were first developed in the problem of acetate activation, in which the classical scheme of ATP utilization seemed quite satisfactory in the case of certain bacteria as described above, but seemed inadequate in the case of animal tissues. The problem was doubly difficult because it was shown that in contrast to the bacterial system there was one enzyme instead of two, and pyrophosphate was released instead of phosphate. In the one-enzyme mechanism it has come to be generally agreed that a two step mechanism is involved and that the enzyme forms compounds with the re-

actants with the result that ternary collisions are not required. The formation of an enzyme-substrate compound was partly an outgrowth of the unexpected difficulties with the acetate system when it proved impossible to fit the animal tissue extracts into the classical pattern that was satisfied by the bacterial systems. In 1946 Lipmann[61] concluded his review covering the failure to demonstrate acetyl-phosphate in animal tissue extracts as follows: "In the theory of the mechanism of acetylation, apparently contradictory facts are harmonized by partial renunciation of the classical concept of a rigorous subdivison of enzymic chains into isolatable intermediaries". The 1946-1956 period is well summarized by Berg[10] who studied acetate activation in detail. Acetyl-phosphate does not appear in the following equations. Acetyl CoA is formed but apparently only one enzyme is involved, and ATP utilization leads to AMP and PP, not ADP and P. In any report in which one enzyme appears to carry out 2 reactions we have to realize that further fractionation may reveal 2 or more enzymes, but in the systems described in this and the next section, all available evidence suggests a single enzyme. In the present example the enzyme is referred to as aceto-CoA-kinase, which is believed to react in 2 steps as follows:

$$ATP + acetate + E(enzyme) \rightleftharpoons E\text{-}AMP\text{-}Ac + PP \qquad (39)$$

$$E\text{-}AMP\text{-}Ac + CoA \rightleftharpoons Acetyl\text{-}CoA + AMP + E \qquad (40)$$

Sum: $\quad ATP + Acetate + CoA \rightleftharpoons Acetyl\text{-}CoA +$
$$AMP + PP \qquad (41)$$

This reaction may be compared with reaction 22.

The occurrence of the reaction as formulated included the study of isotopic exchange and constitutes a catalog of methods in this type of work:

(1) The exchange of $P^{32}P^{32}$ and ATP required acetate.
(2) Synthetic adenyl acetate was readily converted to ATP in the presence of PP and to acetyl CoA when CoA was present.
(3) The accumulation of acethydroxamic acid, PP, and AMP in the presence of hydroxylamine was independent of CoA.
(4) The exchange of AMP with ATP required both acetate and CoA.
(5) The exchange of acetate with acetyl CoA required both AMP and PP.

This type of group activation is apparently a very widespread phenomenon and a comparable series of experiments originated with the work of Hoagland on amino acid activation. Again the reports by Berg[11] are instructive and provide a useful survey of earlier literature by Hoagland, Lipmann and others, as well as data of great significance.

The evidence in favor of amino acid activation as a one-enzyme pyrophosphate-release mechanism is as follows:

1. No exchange of $P^{32}P^{32}$ with ATP in the absence of amino acid.
2. In the presence of hydroxylamine, equal amounts of amino acid hydroxamate, AMP, and PP accumulated.
3. Free AMP was not formed.
4. O^{18} from the carboxyl group of the amino acid was transferred to the phosphate of AMP in the presence of hydroxylamine.

The above evidence provides support for the fol-
lowing reaction

$$E + ATP + \text{amino acid} \rightleftharpoons \text{amino acyl-AMP-E} + PP \quad (42)$$

In the product the amino acid is in anyhydride
linkage with the P of AMP. The symbol for E is
included because of the tight complex formed,
which apparently does not break up in the absence
of amino acid acceptor. The latter may be arti-
ficial, as hydroxylamine, or natural as soluble
RNA, which corresponds to the Y in equation 22,
thus reaction 42 is followed by

$$\text{amino acyl-AMP-E} + sRNA \rightleftharpoons \text{E-sRNA-aa} + AMP \quad (43)$$
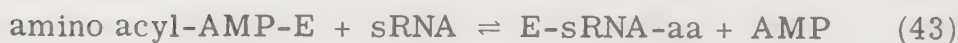
It appears that further transfer of the amino acid
to peptide linkage may occur in a reaction:

$$XY + Z \rightarrow XZ + Y \quad (44)$$
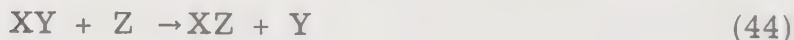
where X is amino acid, Y is soluble RNA, Z is
ribosomal RNA-protein, and XZ represents the
incoming amino acid in peptide linkage, thus:

$$\text{E-sRNA-aa} + \text{ribosome} \xrightarrow{GTP} \text{E-sRNA} + \text{ribosome-aa} \quad (45)$$

followed by

$$\text{E-sRNA} \rightleftharpoons E + sRNA \quad (46)$$

The sum of reactions 42, 43, 45, and 46 is

$$ATP + \text{amino acid} + \text{ribosome} \rightarrow \text{ribosome-aa} + AMP + PP \quad (47)$$

If we assume that this is the major pathway,
and that a side reaction can occur as follows:

$$\text{E-sRNA-aa} \rightleftharpoons E + sRNA\text{-aa} \quad (48)$$

it would not be surprising if under the proper
circumstances the incorporation of labeled amino
acids into ribosomal protein could exceed the in-
corporation into sRNA, without abandoning the
concept of sRNA as a coenzyme, with a specific
enzyme and sRNA for each amino acid. Re-
action 45 should be a logical step to be affected
by a repressor and if blocked by a repressor,
might lead to an increase in the extent of reaction
48, while a substrate might de-repress by occu-
pying sites common to the repressor action but
accelerating reaction 45.

Parenthetically it may be noted that parallel stu-
dies in other laboratories indicate that there may
be instances of amino acid activation by one- or
two-enzyme phosphate-release since a system
exchanging ADP with ATP has been found[9]. Thus
it appears that both acetate and amino acid activa-
tions may proceed by phosphate or pyrophosphate
release, presumably in different organisms, but
it must be pointed out that there is no logical de-
man that prevents both mechanisms from occur-
ring in the same organism, and no compelling
experimental evidence either.

It may also be noted here that reaction 44 also
applies to acetate activation since XY corresponds
to acetyl-CoA and Z to a wide variety of acetyl
acceptors. In the case of the acetate story there
appear to be specific enzymes for each acetyl
acceptor[23], but in the case of the ribosomes both
functions are served.

E.   *One-enzyme phosphate-release*

To continue our emphasis on alternative mechan-
isms of ATP utilization, we come to the final
category in the present discussion. With the re-
alization that the two-enzyme phosphate release
mechanism was not unique, there was a period

when pyrophosphate release held the center of the stage, and it was seen that this could come about by the successive action of two enzymes or more recently, by a one enzyme mechanism, as outlined above. In parallel with these studies it became evident that certain reactions would not fit into any of the above categories, and this was realized and decribed in several papers from the laboratories of Buchanan[18, 34] and Bloch[103]. Buchanan described the synthesis of glycinamide ribotide (GAR) from glycine, ATP and phosphoribosylamine)PRA) as follows (compare Chapter VI):

$$ATP + glycine + PRA \rightarrow GAR + ADP + P \qquad (49)$$

All attempts to fractionate the reaction into 2 steps catalysed by separate enzymes were unsuccessful and it has been concluded that a single enzyme is involved. According to Hartman and Buchanan[34] the mechanism is similar in a number of cited examples, in which there is a hydrolysis of the terminal pyrophosphate bond in ATP and a concommitant removal of water from reactants corresponding to X and Y in equation 23, or glycine and PRA in equation 49. "The same oxygen atom which is removed in the dehydration of the reactants is used to perform the hydrolysis of the ATP. A covalent bond is thereby formed between the two Reactants A and B".[34]

Characteristics of the class are:

1. Products of ATP utilization are ADP and P.
2. No evidence for more than one enzyme.
3. Carboxylactivations are demonstrated by the formation of hydroxamic acids in the presence of hydroxylamine and ATP.
4. Arsenolysis of the products requires ADP.
5. P^{32} exchange into ATP requires both carboxyl and amino reactants.

6.   Formation of either carboxyl or amino re-
     actant by reversal or exchange reactions
     requires both ADP and phosphate or arsenate.

The findings preclude mechanisms involving the
formation of covalent enzyme-substrate com-
pounds or of free carboxyl phosphate intermedi-
ates.   Buchanan and Hartman[18] refers to this
type as simultaneous or "concerted reactions"
but we prefer to call them the one-enzyme phos-
phate-release type to emphasize the symmetry
of the 4 known mechanisms, and to avoid calling
any of the other types "disconcerted".   The dis-
cussion of the one-enzyme reactions[18] is outstand-
ing and should be consulted for further insight into
the nature of these reactions.

It is of interest that in many synthetic reactions
with a series of sequential steps, the driving
mechanism may involve phosphate release in one
step and pyrophosphate release in another step,
or ATP in one step and another triphosphate in
a following step.   Thus in the reaction

$$X + Y + ATP \rightarrow XY + ADP + P \xrightarrow{+ ATP + Z} XYZ + AMP + PP \quad (50)$$

The tendency to form XYZ might be greater than
it would be if the second step also formed ADP
and P.

An example of such a sequence is evident in the
report by Strumeyer and Bloch[103] who note that
in the first step of glutathione synthesis, glutamyl
cysteine synthetase releases P and ADP, while
the second step, studied by Bates and Lipmann,
led to the formation of an sRNA derivative and
presumably liberated pyrophosphate.

In the discussion of the four ways for ATP utili-

zation we have set up descriptive titles not employed by any other reviewer but it is believed they should be most helpful to students since no other systematic nomenclature is accepted or available. The review by Bock[13] is a most helpful supplement to the present discussion and includes many ideas and references to physico-chemical considerations, especially the role of chelation by magnesium and other metals, that are not presented in the above.

It is worth emphasizing that the processes of nucleic acid synthesis include both phosphate release and pyrophosphate release (Chapter VII) and that nucleic acid synthesis must be considered as an alternative metabolic pathway that is in competition with the coenzyme functions of the nucleotides as described in this chapter.

Finally, the limitless array of the reactions participated in by the nucleotide coenzymes, of which only a small fraction have been discussed here, involves the same basic units that are required for nucleic acid synthesis, and reminds us of the truly fundamental importance of these building blocks in life processes. We are challenged by the realization that probably no single mind can encompass the details of all the reactions in which these fundamental units participate, and further impressed by the thought that many additional coenzymes are not nucleotides. Thus we are compelled to attempt generalizations and categories of reactions and of structures in the hope that we can build in terms of principles if not in terms of infinite detail.

This chapter concludes Volume I of Nucleic Acid Outlines, which has been concerned with the chemistry and metabolism of the nucleic acids

and nucleotides.   Volume II is intended to cover the function of the nucleic acids and again the wealth of biological detail forces us to proceed in terms of generalizations wherever possible.

REFERENCES

1.   *ABRAHAM.   Biochem. J. 33:543 (1939).*

2.   *AGRANOFF, BRADLEY and BRADY. J. Biol. Chem. 233:1077 (1958).*

3.   *ARMSTRONG, BADDILEY, BUCHANAN and CARSS. Nature 181:1692 (1958).*

4.   *BADDILEY and BLUMSON. Biochem. Biophys. Acta 39:376 (1960)*

5.   *BADDILEY and BUCHANAN. Quart. Rev. Chem. Soc. 12:152 (1950).*

6.   *BADDILEY, BUCHANAN, CARSS, MATHIAS, and SANDERSON. Biochem. J. 64:599 (1956).*

7.   *BADDILEY, BUCHANAN, and GREENBERG. Biochem. J. 66:51P (1957).*

8.   *BEINERT in Boyer et al[15] vol. 2 pp. 339-416; 426 references.*

9.   *BELJANSKI and OCHOA. Proc. Nat. Acad. Sci. 44:1157 (1958).*

10. *BERG. J. Biol. Chem. 222:991, 1015, 1022 (1956).*

11. *BERG. J. Biol. Chem. 233:601, 608 (1958).*

12. *BERGKVIST and DEUTSCH. Acta. Chem. Scand. 7:1307 (1953). 8:1889 (1954).*

13. *BOCK in Boyer et al[15], vol. 2, pp. 3-38; 169 references.*

14. *BORKENHAGEN and KENNEDY. J. Biol. Chem. 227:951 (1957).*

15. *BOYER, LARDY and MYRBÄCK. The Enzymes, 2nd Ed. Academic Press, New York, 1960.*

16. *BROWN, G. M. J. Biol. Chem. 234:370 (1959).*

17. *BRUMM, POTTER and SIEKEVITZ. J. Biol. Chem. 220:713 (1956).*

18. BUCHANAN and HARTMAN. Adv. Enzymol. 21:199
         (1959).
19. CABIB and LELOIR. J. Biol. Chem. 206:779 (1954).
20. CANTONI and DURELL. J. Biol. Chem. 225:1033
         (1957).
21. CAPUTTO, LELOIR, CARDINI and PALADINI.
         J. Biol. Chem. 184:333 (1950).
22. CARTER and COHEN. J. Biol. Chem. 222:17 (1956).
23. CHOU and LIPMANN. J. Biol. Chem. 196:89 (1952).
24. COHN. J. Am. Chem. Soc. 72:1471 (1950), and
         CHARGAFF and DAVIDSON, Vol. I, p. 211.
25. COHN and VOLKIN. Nature 167:483 (1951).
26. COLOWICK and SUTHERLAND. J. Biol. Chem.
         144:423 (1942) .
27. COOPER and LEHNINGER. J. Biol. Chem.
         219:489 (1956).
28. DAVIDSON. Biochim. Biophys. Acta 33:238 (1959).
29. DOERY. Nature 177:381 (1956).
         ibid 180:799 (1957).
30. EMBDEN. Arch. Exptl. Path. Pharmakol. 167:50
         (1932).
31. EMBDEN and LAQUER. Z. physiol. Chem. 93:95
         (1914).
32. EMBDEN and ZIMMERMAN. Z. physiol. Chem.
         167:114 (1927).
33. GREGORY and LIPMANN. J. Biol. Chem. 229:1081
         (1957).
34. HARTMAN and BUCHANAN. J. Biol. Chem. 233:456
         (1958).
35. HAYNES, J. Biol. Chem. 233:1220 (1958) and HAYNES,
         KORITZ, and PERON. J. Biol. Chem. 234:1421
         (1959).
36. HENDERSON and LE PAGE. Chem. Rev. 58:645
         (1958).
37. HERBERT and POTTER. J. Biol. Chem. 222:453
         (1956).
38. HERBERT, POTTER and TAKAGI. J. Biol. Chem.
         213:923 (1955).
39. HURLBERT. Fed. Proc. 11:234 (1952).
         Fed. Proc. 12:222 (1953).

40. HURLBERT and POTTER. J. Biol. Chem. 195:257
          (1952); 209:1 (1954).
41. HURLBERT, SCHMITZ, BRUMM and POTTER.
          J. Biol. Chem. 209:23 (1954).
42. JAENICKE and LYNEN in Boyer et al[15] vol. 3
          tentative contents.
43. KAPLAN in Boyer et al[15], vol. 3 tentative contents.
44. KATUNUMA. Arch. Biochem. Biophys. 76:547 (1958).
45. KELLER and ZAMECNIK. J. Biol. Chem. 221:45
          (1956).
46. KELLERMAN. J. Biol. Chem. 231:427 (1958).
47. KENNEDY in Boyer et al[15], vol. 2 pp. 63-74; 34
          references.
48. KENNEDY and WEISS. J. Biol. Chem. 722:193
          (1956).
49. KIESSLING and MEYERHOF. Biochem. Z. 296:410
          (1938).
50. KORNBERG. J. Biol. Chem. 176:1475 (1948).
                              182:779 (1950),
51. KORNBERG in McElroy and Glass. The Chemical
          Basis of Heredity, pp. 579-608, Baltimore,
          1957.
52. KORNBERG and PRICER. J. Biol. Chem. 186:557
          (1950).
53. KORNBERG and PRICER. J. Biol. Chem. 191:535
          (1951).
54. KURAHASHI, PENNINGTON, and UTTER. J. Biol.
          Chem. 226:1059 (1950).
55. LELOIR and CARDINI. J. Am. Chem. Soc. 79:6340
          (1957).
56. LELOIR and CARDINI in Boyer et al[15]. Vol. 2,
          pp. 39-62; 134 references.
57. LePAGE. J. Biol. Chem. 226:135 (1957).
58. LePAGE and UMBREIT. J. Biol. Chem. 148:255
          (1943).
59. LIEBERMANN. J. Am. Chem. Soc. 77:3373 (1955).
60. LIEBERMANN. J. Biol. Chem. 223:327 (1956).
61. LIPMANN, in Nord and Werkman. Adv. Enzymol.
          1:99 (1941).
          6:231 (1946).

62. LIPMANN. J. Biol. Chem. 155:55 (1944).
63. LIPMANN. Science 120:855 (1954).
64. LIPMANN. Proc. Nat. Acad. Sci. 44:67 (1958).
65. LIPTON, MORELL, FRIEDEN, and BOCK. J. Am.
          Chem. Soc. 75:5449 (1953).
66. LOHMANN. Biochem. Zeit. 282:109 (1935).
67. LOHMANN. Naturwissenschaften 17:624 (1929).
          Biochem. Zeit. 282:120 (1935).
68. LYNEN, REICHERT and RUEFF. Ann. Chem.
          574:1 (1951).
69. MARRIAN. Biochim. Biophys. Acta. 13:278 (1954).
70. MARTONOSI. Biochem. Biophys. Res. Comm.
          2:12 (1960).
71. MC ELROY and GLASS. Phosphate Metabolism.
          Vols. I and II. Johns Hopkins Press
          (1951, 1952).
72. MOSLEY and CAPUTTO. J. Am. Chem. Soc.
          80:4746 (1958).
73. MUNCH-PETERSON. Arch. Biochem. Biophys.
          55:592 (1955).
74. OHLMEYER. J. Biol. Chem. 190:21 (1951).
75. OKAZAKI. Biochem. Biophys. Res. Comm.
          1:34 (1959).
76. OKAZAKI, OKAZAKI and KURIKI. Biochim. Biophys.
          Acta. 33:289 (1959).
              38:384 (1960).
77. OSTERN. Biochem. Z. 270:1 (1934).
78. PARK. J. Biol. Chem. 194:877, 885, 897 (1952).
79. PAULUS and KENNEDY. J. Biol. Chem.
          235:1303 (1960).
80. PONTIS and BLUMSON. Biochim. Biophys. Acta
          27:618 (1958).
81. POTTER. Medicine 19:441 (1940).
82. POTTER in Nord and Werkman. Adv. Enzymol.
          4:201 (1944).
83. POTTER and BUETTNER-JANUSCH. J. Biol. Chem.
          233:462 (1958).
84. POTTER, SCHLESINGER, BUETTNER-JANUSCH,
          and THOMPSON. J. Biol. Chem. 226:381 (1957).

85.   REICHARD and RUTBERG. Biochim. Biophys. Acta.
          37:554 (1960).
86.   REYNAFARJE and POTTER. Cancer Research
          17:1112 (1957).
87.   ROBBINS and LIPMANN. J. Biol. Chem. 233:681,
          686 (1958).
88.   ROBBINS, TRAUT and LIPMANN. Proc. Nat. Acad.
          Sci. U. S. 45:6 (1959).
89.   ROSE, GRUNBERG-MANAGO, KOREY and OCHOA.
          J. Biol. Chem. 211:1954 (1954).
90.   SACKS. Biochim. Biophys. Acta. 16:436 (1955).
91.   SANADI, GIBSON, AYENGAR, and JACOB. J. Biol.
          Chem. 218:505 (1956).
92.   SCHLENK. J. Biol. Chem. 146:619 (1942).
93   SCHLENK and VON EULER, Naturwissenschaften
          24:794 (1936).
93.   SCHMIDT, G. Z. physiol. Chem. 179:243 (1928).
94.   SCHMITZ, HURLBERT and POTTER. J. Biol. Chem.
          209:41 (l954).
95.   SCHMITZ, POTTER, HURLBERT and WHITE.
          Cancer Research 14:66 (1954).
96.   SCHNITGER, PAPENBERG, GANSE, CZOK, BÜCHER
          and ADAM. Biochem. Zeit. 332:167 (1959).
97.   SHAW. Biochem. J. 66:56P (1957).
98.   SIEKEVITZ and POTTER: J. Biol. Chem. 215:221,
          237 (1955).
99.   SRIBNEY and KENNEDY. J. Am. Chem. Soc.
          79:5325 (1957).
100. STADTMAN. J. Biol. Chem. 196:527, 535 (1952).
101. STROMINGER. Biochim. Biophys. Acta. 16:616
          (1955)
102. STROMINGER and SCOTT. Biochim. Bi phys. Acta.
          35:552 (1959).
103. STRUMEYER and BLOCH. J. Biol. Chem. 235:PC27
          (1960).
104. SUGINO. J. Am. Chem. Soc. 79:5074 (1957).
105. SUTHERLAND and RALL. J. Biol. Chem. 232:1065,
          1077 (1958).
106. TSUYUKI and IDLER. J. Am. Chem. Soc. 79:1771
          (1957).

107.   UTTER in Boyer et al[15] pp. 75-88; 69 references.
108.   UTTER, KEACH and NOSSAL. Biochem. J. 68:431
          (1938).
109.   WANG, SCHUSTER and KAPLAN. J. Biol. Chem.
          206:299 (1954).
110.   WARBURG, CHRISTIAN and GRIESE. Biochem. Z.
          282:157 (1935).
111.   WARBURG and CHRISTIAN. Biochem. Zeit.
          298:150 (1938).
112.   WEISS, SMITH and KENNEDY. J. Biol. Chem.
          231:53 (1958).
113.   WILLOUGHBY and WAISMAN. Cancer Research
          17:942 (1957).